A Symposium on

COPPER METABOLISM

Edited by

William D. McElroy and Bentley Glass

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COPPER METABOLISM

A SYMPOSIUM ON
ANIMAL, PLANT AND SOIL RELATIONSHIPS

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COPPER METABOLISM

A SYMPOSIUM ON
ANIMAL, PLANT AND SOIL RELATIONSHIPS

sponsored by

THE
McCOLLUM-PRATT INSTITUTE
OF
THE JOHNS HOPKINS UNIVERSITY

Edited by

WILLIAM D. McELROY AND BENTLEY GLASS

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PREFACE

A Symposium on Copper Metabolism was held at Johns Hopkins University under the sponsorship of the McCollum-Pratt Institute on June 14, 15 and 16, 1950. This publication consists of the papers and discussions presented at these meetings.

Copper was selected as the primary topic in order to allow a more thorough coverage of the numerous studies which have been conducted in the fields of soil science, animal and plant nutrition and biochemistry. However, nutritionally the micronutrients cannot always be considered individually. This fact is brought out by several papers in the present symposium. We hope that future symposia will further emphasize the important interrelationships of the micronutrients.

In the selection of speakers an attempt was made to bring together those specialists who, on the basis of their own research, best represented a given area of investigation. In addition to the speakers, a small group of scientists from various fields was asked to participate in the symposium. Thus an opportunity was afforded for a greater exchange of ideas in the various fields of micronutrient research related to copper. It is hoped that the published papers and discussions will be of value to those interested in the general field of micronutrients.

The McCollum-Pratt Institute is indebted to many specialists in the field for their valuable suggestions in planning the Symposium. Thanks are also due to the Scientific Advisory Committee of the Institute for their suggestions and assistance.

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THE FORMATION OF COPPER COMPLEXES

ALSOPH H. CORWIN
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EVERYONE INTERESTED in micronutrients is aware of the contribution which the analytical chemists have made to the field. By investigating vegetable ash they have established which mineral constituents are present and have given the biologists those leads which it is necessary to follow to determine the influence of these materials on plant nutrition and health. They have then provided means, more or less satisfactory, for following the amounts of the micronutrients in various soils and in isolated portions of plant materials, and have thus provided useful tools for following the distribution of these substances. In addition to these major services, numerous others could be cited.

I believe it to be true that biologists are somewhat less aware of the part which biochemists trained in the techniques of organic reaction mechanisms might play in a field such as that of the micronutrients. This is undoubtedly because most of the contributions which can be made from this field are still in the future instead of being accomplished facts, and many of them will require further exploratory biological experimentation as a necessary background for the initiation of sound research programs. It is my purpose here to attempt to point out some of the contributions which might be made to the field by chemists, choosing the reactions of copper as the focal point of attention for purposes of illustration.

Let us suppose that the analytical chemist has established the presence of a metallic constituent such as copper in the ash of plants. A plant physiologist would then be in a position to experiment with plant diets deficient in this element and to determine the effect of its exclusion upon the metabolism of a large variety of plants. He would then be able to proceed with diagnostic and therapeutic measures, and one practical goal would have been achieved.

To secure a greater degree of control, much more knowledge would be essential. A plant biochemist taking this information would be in a position to search for the active enzymatic constituent of the living tissues in which the metallic element functions. If he were successful in finding a suitable assay method for the enzymatic action in question, he would, in most cases, be able to isolate the active enzyme in pure form. He would then be in a position to study its action in more detail, to learn the nature of materials which poison it, to find what substances it acts upon, to find where it fits into a biological chain of synthesis or degradation, to study the effects of other metals in activating or deactivating it, and in general to provide a wealth of background information for the intelligent handling of substances concerned with its most effective functioning. Thus the stage would be set for the achievement of another practical goal.

It is approximately at this point that the biochemist who leans to the organic chemical approach would be able to start his contributions. If he knew the nature of the substrate for the enzymatic action in question, he would be able to modify this chemically and study the effectiveness of the enzyme in dealing with the modifications. He would thus be in a position to learn something about the shape of the active part of the enzymatic molecule and the nature of its chemical interaction with the substrate. On the side of the enzyme itself, he would be in a position to make structural investigations on the active group, to learn the nature of its combination with the metallic element under investigation, and finally to undertake the synthetic production of model substances to duplicate one or more of the properties of the naturally occurring material. As his understanding of structure, activity, and their interrelationships grew, he could hope to achieve a goal which has so far eluded us, the production of an entirely synthetic material capable of duplicating the action of a naturally occurring enzyme. Having achieved this goal, he would be in a position to modify the activity of his synthetic enzyme model by modifying its structure and to substantiate his hypotheses concerning the relationships between chemical structure and biological activity.

It would be useless to speculate about the degree of control over plant physiology and pathology that knowledge of the type just indicated would be capable of yielding. It is certain that such information would give much more precise control than is now possible over therapeutic measures to be taken on soils. Substances capable of neutralizing the effects of overdosage with metallic elements would become available and compounds of micronutrient elements less prone to produce toxic effects by overdosage would certainly result. This would greatly broaden our knowledge of the field which we might call "soil chemotherapy." Past experience indicates that the most useful applications of this type of knowledge, however, would be those that we cannot now foresee.

In requesting a brief review of the chemistry of copper to provide a background for a biological discussion, the organizer of this symposium assigned a peculiarly difficult task. Our chemical knowledge of the copper-containing materials having biological activity is so meager as to provide the chemist with but the slimmest toehold for a pointed discussion. In this situation, two methods of approach are open. The first is descriptive. If this were adopted, a broad survey of the chemistry of copper complexes could be given, in the hope that it would later be discovered that the biologically active compounds resemble some known compound to a sufficient degree to permit attention to be withdrawn from the 999 substances that did not fit and be focused on the one that did. It seems to me that even this long chance would probably fail. For example, the hemocyanins are copper compounds which are capable of combining reversibly with oxygen. Our known copper complexes do not have this power. The chances of finding a known compound without this power whose chemistry could serve as a model for that of the hemocyanins seems remote. Because of the unfavorable nature of this approach, I shall not adopt it.

The second method of approach is mechanistic. We may study the forces at work in various types of chemical combination and learn something of how their interactions are capable of yielding varied results under varied circumstances. While we may not be assured that all the forces which are at work in the biologically interesting cases have yet been catalogued, we may have complete assurance that some of the forces at work are now understood, and we may see how their combinations and extensions could lead to the production of new materials with unique properties.

The field of complex compounds was first exploited systematically by A. Werner (10), over fifty years ago. Pioneering studies in the field were also made by P. Pfeiffer (6). Numerous modern investigators have contributed to our understanding of the forces which determine the phenomena of the chemistry of complex reactions. Parts of this work will be reviewed.

The first force which we shall discuss may be called " polarity " because of the fact that the term is familiar to biologists. The polarity of the chemist is fundamentally related to the positive and negative electrostatic charges of the physicist and is responsible for the familiar phenomenon of the periodicity of the elements, as well as for the somewhat less familiar phenomenon of atomic size. We shall be interested in both of these phenomena in connection with our investigation of the chemistry of copper.

If we imagine a central positively charged particle attracting a group of negatively charged particles which are constrained from colliding with the positive nucleus, the resultant figure will have a shape determined by the resultant of the attractive force of the nucleus and the respective repelling forces of the negatively charged particles. A two-dimensional model of such a system was investigated by R. W. Wood (11) in 1898. The interesting feature of this model is that regular polygons are formed, up to a certain point. As the rings surrounding the central nucleus expand, however, the negatively charged particles tend to recede too far from the center for stability to be maintained. At this point one of them wanders into the center, and the remainder start to build a smaller ring about it. The building process can then be repeated until a third period is formed, and so on. From this model one learns that the resultants of these forces of opposite polarity are not rings of increasing size but instead a series of rings of differing numbers of constituents, resembling in this respect the rows of the periodic table.

The radius of the outer ring in any case will be determined by the magnitude of the oppositely polarized forces at work. In the case of the chemical elements in the periodic table, the addition of each valence electron is accompanied by the addition of a single positive charge on the nucleus. Thus the central force increases as we move up the periodic table. The effect of this on the atomic radius is most readily grasped by reference to Pauling's so-called "isoelectronic series" (4), that is, the series across the periodic table in which the number of outer electrons is maintained constant in each row while the nuclear charge increases, thus systematically increasing the overall charge on the resulting ion. It will readily be seen that the size of the resulting ion will diminish systematically along each row. For example, in the first row the values calculated are Li^+, 0.60; Be^{++}, 0.31; B^{3+}, 0.20; C^{4+}, 0.15; N^{5+}, 0.11; O^{6+}, 0.09; F^{7+}, 0.07.

The sizes to be expected when the normal complement of electrons is permitted to remain on each atom are not so readily apparent. For our present purposes it is sufficient to know that the nature and magnitude of the forces of attraction and repulsion at work are such that the forces of attraction predominate increasingly as one proceeds along a row of the periodic table, so that each succeeding element has a smaller radius than the preceding one. Thus in the series C, N, O, F, the covalent radii in Ångstrom units are: C, 0.77; N, 0.70; O, 0.66; F, 0.64.

Each successive ring of electrons that is added to the atom increases its radius, as would be anticipated. Thus the atomic radius increases as one goes down a column of the periodic table. For instance, in the halogen family the radii as one goes down the column are: F, 0.64; Cl, 0.99; Br, 1.14; I, 1.33.

These relationships can be combined to give a general, rough relationship which is useful, namely, that atomic radius increases in the periodic table when one proceeds from upper right to lower left.

Atomic radius by itself has an important bearing on the formation of complexes. If we have a small central atom and wish to place around it a number of large atoms, it immediately becomes apparent that the number that can be introduced is limited by the size of the outer atoms. If too many large atoms were introduced, they would tend to overlap and crowd each other away from the center. This principle was recognized by Magnus (3). Limiting radius ratios can be calculated by inscribing outer spheres of suitable diameters tangent to the central coordinating sphere at the proper angles and finding the interferences graphically (9). For symmetrical distribution of three groups around a central atom we find a radius ratio of 0.155. This means that if the outer spheres are approximately six times as large as the central sphere three of them can just be fitted on to the surface. If they are larger than this, only two would fit. For symmetrical distribution of four groups, the limiting radius ratio is 0.225. Roughly, this means that outer spheres must be smaller than four times the radius of the central sphere to permit four of them to cluster in it. For symmetrical distribution of six groups, the radius ratio figure is 0.414, meaning that spheres only two and a half times as large as the central sphere can be accommodated in this arrangement. For eight atoms around a central sphere, the limiting radius ratio is 0.732, meaning that in this distribution the outer spheres can only be approximately one and a third times as large as the central sphere to be accommodated. Thus we see that the common sense rule which we would expect is followed. The larger the groups you wish to place next to the central atom, the fewer such groups can be accommodated.

Stripped of its valence electrons, copper in the form of its cuprous ion has a radius of 0.96 Å. With its bonding electrons in place, the cuprous atom has a radius of 1.35 Å. Each of these figures represents an atom of moderate size. This size turns out to be sufficient, in comparison with the largest in the periodic table, that the radius ratio effect would never limit the number of atoms that could be placed around copper to a number below four, and only in extreme instances would any limitation be placed on having six groups around the copper. Since the ordinary coordination number of copper is four and only few compounds with higher coordination are known, this means that copper is large enough to coordinate about itself as many atoms of as large size as its other properties will permit. This gives us an important element of freedom in dealing with copper complexes.

The next important consideration with respect to coordination complexes is the overall shape of the complex. An important factor in determining this shape is the included angle between the bonds of the central complexing atom. In general, it may be stated that the bonds, being made up of electrons which have a like charge, tend to repel each other and to assume positions in space as far apart as possible. The exceptions to this statement will appear as we proceed (8a).

If we have a central atom with only two atoms linked to it, the included angle between the bonds will tend to be 180°. It is probable that most of the copper complexes with a coordination number of two, such as the diammines, have this structure.

The next common coordination number is four. The arrangement which permits greatest separation of four groups on a spherical surface is the tetrahedral, with included angles of approximately 109.5°. This is the bond angle found in carbon compounds, such as methane, with four substituent atoms on the central atom. It is also the arrangement in the cuprous complexes, which have an electronic structure essentially similar to that of the carbon compounds.

An interesting case is that of ammonia. This molecule might conceivably be arranged with all four atoms in the same plane, or it might be pyramidal, with the three hydrogens in one plane and the nitrogen in another. In the latter case the molecule could be regarded as a truncated tetrahedron, or as a tetrahedron in which the top point is occupied by an electron pair instead of by another atom. In fact, it is found that the latter case is the correct one—ammonia is pyramidal.

Another interesting case is that of water. At first it might appear that the bond angles should be 180°. If we consider water to be similar to the case of ammonia, however, we soon perceive that it also should be a truncated tetrahedron with points occupied by electron pairs and two by hydrogen atoms. Again this is the structure found. From these cases we may conclude that electron pairs should be taken into account in deciding on bond angles much as if the positions they occupy were occupied instead by atoms.

Another case that we need to consider is that in which six atoms surround the central atom. In this case again, the bonds will seek the positions in space which permit them to be farthest apart. This turns out to be the octahedral arrangement (Fig. 1). If a sphere is placed at the center of a regular octahedron and bonds are drawn from it to each of the six corners of the octahedron, it will be found that the included angles between bonds are all 90°. This is the case in the well-known ferro- and ferri-cyanide complexes. This bond type also appears in the 6-covalent cupric ammine complexes.

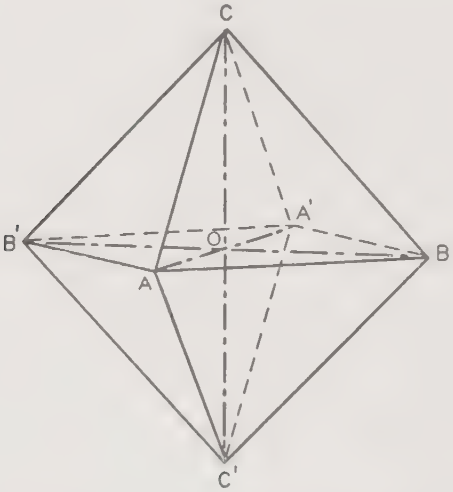

Fig. 1. Octahedral Arrangement of Atoms as in Cupric Ammine Complexes.

The last case which we shall consider is that of the 4-covalent cupric complexes. On examination it will be found that this bond type bears closer resemblance to the ferrocyanides than it does to the cuprous complexes. It has a relationship to the ferrocyanides similar to the relationship between methane and water. If we look upon the cupric ammonia complex as an octahedron truncated to a plane, with an electron pair in the top position and a single electron at the bottom, we have an arrangement in which the central atom is at the center of a square and the included angles between the bonds are 90°. This is the arrangement actually found in the cupric complexes.

We are now in a position to combine two of the pieces of information which we have obtained. If we consider a five-membered ring in which each of the atoms is carbon, with a normal bond angle of 109.5°, we see that there is very little strain in bending all the bonds to the angle of 108° required to form a regular pentagon. If we now substitute a much larger atom, such as copper, for one of the carbons, the effect would be to open up the ring if the bond angles were left unaltered. However, in the case of the cupric complex, the bond angles are altered and in precisely the way required to bring the bonds back toward their original positions. The 90° cupric bond angle almost exactly compensates for the extra size of the copper atom, with the result that the five-membered ring is again extraordinarily stable. This is illustrated by the formation of copper glycinate and the copper complex of ethylene glycol (Fig. 2).

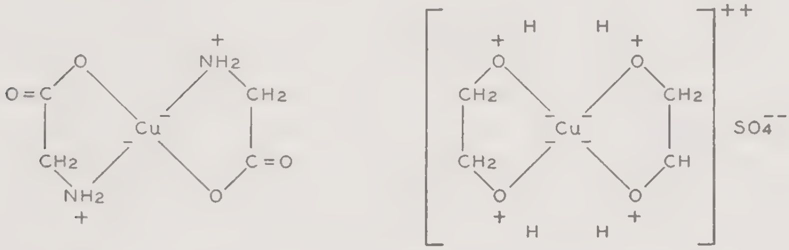

Fig. 2. 90° Cupric Bond Angles in Five-Membered Rings.
Left, Copper glycinate. Right, Ethylene glycol.

We have seen how the interactions of polar forces account for the periodicity of the elements, their sizes, and their bond angles. We shall now examine the effect of polarity as one of the factors influencing the strength of chemical binding and the mutual affinities of atoms in chemical compounds.

It will be recalled that the increasing attractive forces exerted by the nucleus as we go across the periodic table from left to right result in steadily closer approach of the electrons to the nucleus and diminishing atomic radius. This information is of immediate use in the study of chemical affinities.

In biological systems the elements whose affinities for copper will be of greatest interest are nitrogen, oxygen, and sulfur. Let us first consider their affinities for copper in air, that is, in the dry state. We may consider each bond between one of these elements and copper as a tripole with the positive nucleus of the element under consideration at one end, the negative electrons in the middle and the positive nucleus of the copper at the other end. If we now imagine a process by which the positive charge of the first nucleus is increased, it will be seen that the attraction for the electrons will be increased and the repulsion for the copper nucleus will not be increased nearly so much, since it operates at a much greater distance. The net effect of this increase of charge on the nucleus of the element bound to copper will be to increase the strength of binding. This effect is that which is actually observed in going from nitrogen to oxygen. In the dry state, nitrogen has only moderate affinity for copper and oxygen has much, in line with the greater attracting power of the oxygen nucleus.

Let us consider the same process taking place in a column of the periodic table, moving from top to bottom. In this case, it is true that the nuclear charge has increased. However, because of the interposition of extra rings of electrons, the distance from center to circumference has also increased, and the outer electrons are screened from the effect of the nucleus by the inner electrons. The combined effect of distance and screening overbalances the effect of the increased attraction of the nucleus, with the result that electron-attracting power diminishes as we go from top to bottom in the table. We may thus formulate a rough guide to electron-attracting power which will usually work going directly across the periodic table or directly up and down but will not necessarily work when moving diagonally across the table. This rule is that the larger the atomic radius the less the attracting power of the nucleus for valence electrons. In our particular case, this means that the affinity of sulfur for copper, in the dry state, should be less than that of oxygen. This is also found to be true

(1, 2). In general, this relationship may be expected to hold whenever a medium of very low dielectric constant is substituted for air. A biological example would be fat, which has a very low dielectric constant.

When we change to aqueous solutions, these affinity relationships undergo a profound modification. This may be largely charged to the tremendous increase in dielectric constant accompanying the change in medium. The effect of the increase in dielectric constant is to make it progressively easier to separate ions. In air, ions tend to stick together. In water, they tend to move apart. Hence, in water, increasing polarity results in increasing ionization and therefore in diminished affinity, in contrast to the case in air.

A simple illustration of this effect may be found in the formation of the copper-ammonia complexes. Because of the strong electron-attracting power of the oxygen nucleus H_3O^+ is a strong acid, it being the main species responsible for the acidity of water solutions of mineral acids. In losing its proton and thus ionizing, the oxygen of H_3O^+ exerts its electron-attracting power in such a manner as to monopolize an electron pair. In this case, then, electron-attracting power no longer parallels bond strength. In the case of the ammonium ion, the attraction of the nitrogen for its electrons is less than that of oxygen in the hydronium ion, with the result that the ammonium ion does not part with its hydrogen as readily and is thus a weaker acid than the hydronium ion. This is shown by the well-known fact that ammonium chloride is a weaker acid than a water solution of hydrochloric acid. The converse argument is also true. Because of its lesser affinity for the hydrogen ion, just demonstrated, water is a weaker base than ammonia.

Let us apply this information to the case of copper. Just as the stronger base, ammonia, has greater affinity for the positively charged hydrogen ion, so it has greater affinity for the positively charged copper ion. In water solution, then, competition between H_2O and NH_3 for copper will result favorably to NH_3, with ammine formation taking place (8b). As acidity is increased, however, NH_3 is converted to NH_4^+ long before conversion of H_2O to H_3O^+ becomes appreciable. Because the systems under consideration are dynamic, this results in the removal of the reacting species, NH_3, from the scene of action and in conversion of the ammine to the water complex. These relationships account for the well-known fact that copper-ammonia complexes are not stable in acidic solutions, in spite of the relatively large affinity of nitrogen for copper in aqueous media.

While the case of the relationship between oxides and sulfides is not quite so clear as that of the relationship between oxides and ammines, the effect is the same. In air oxides are more stable. The reason once more appears to be the relative polarities. The copper-oxygen bond, being more polar, has a greater tendency to ionize and therefore to dissociate in water solution. The copper-sulfur bond, being less polar, is less affected by the change in medium. The result is that the treatment of copper hydroxide with hydrogen sulfide in water solution converts the copper to the less polar form, copper sulfide.

We thus see that the nature of the medium in which a competitive reaction is run has a profound effect on the outcome. Biological systems have at their disposal media fully as varied as those we have discussed. In inquiring into the complexing ability of copper with biological chemicals, it is necessary to know with some precision the nature of the immediate microscopic surroundings of the complexing reaction site. If this is a fatty medium, oxygen would be expected to have the greatest affinity for the copper. If it were an aqueous medium, sulfur would react most readily, nitrogen next, and oxygen last.

The next question which we must ask in considering the complexing ability of copper is: how many groups may coordinate with copper? The theoretical answer to this question is found by reference to the so-called "rare gas rule"; the practical answer, which is different, by a search of the literature.

The rare gas rule states that the maximum covalence of an element is determined by the number of electrons required to fill its orbitals to that of the next higher rare gas. In certain cases, covalence less than this may be found. Higher covalences are not expected, however. As examples C, N, O, and F all fill their orbitals up to the value for neon, requiring 4, 3, 2, and 1 electrons, respectively. The last four elements of the second row fill their orbitals up to the value of argon. In the row to which copper belongs, maximum covalence is obtained by filling the orbitals up to the value of krypton. In the cuprous state, this is accomplished by bonding with a total of four groups. Values of two, three, and four are all found frequently; but values higher than four apparently do not occur. In the cuprous complexes, then, the rare gas rule seems at present to be a safe guide to the maximum complexing power of the element (8c).

The case of the cupric complexes is different. The maximum number to be expected under the rare gas rule is five. In practice, two occurs in a number of compounds; three occurs rarely if ever; four is by far the most common; five occurs rarely; but the exceptional cases are the few compounds containing six attached groups around the copper (5, 7). The rare cases of five and six covalent cupric complexes should be of especial interest to the biochemist, since they have been studied so little as to carry some promise of exceptional properties.

The final effect to which I wish to direct attention is the formation of simultaneous links to the same organic molecule, the so-called " chelation " phenomenon. It is obvious that two links will be stronger than one and that a compound which has to be hit twice as hard to break it will be more stable than a compound with a single link. What is not immediately obvious, however, is that the stabilization obtained by this type of multiple linkage is much greater than would be predicted on the simple basis just presented. This is because blows with twice a given amount of energy occur with a very much smaller frequency than one-half. If we imagine a single linkage so weak that its half-life expectancy is only about a minute at room temperature, a double link of the same strength would have a half-life expectancy of nearly a quarter of a million years, providing that the temperature were not raised and that no other effect intervened.

All of the effects which have been mentioned are combined to give great stability in the case of the copper complexes of porphyrins

(Fig. 3). In these substances, the hole in the center of the large ring is of the right size to accommodate a copper atom, the nitrogen atoms are held rigidly at 90° angles, just right for the cupric atom, the complex-forming atoms are nitrogen, which has great affinity for copper in aqueous solutions, and chelation is compounded four times to yield an extraordinarily stable complex. The affinity of porphyrins for copper is so great that they pull it out of copper sulfide. If one should attempt to measure the absorption spectrum of a porphyrin in a brass tube, the porphyrin would be capable of pulling the copper out of the brass. These copper complexes are so stable that it takes most of the power of concentrated sulfuric acid to break them.

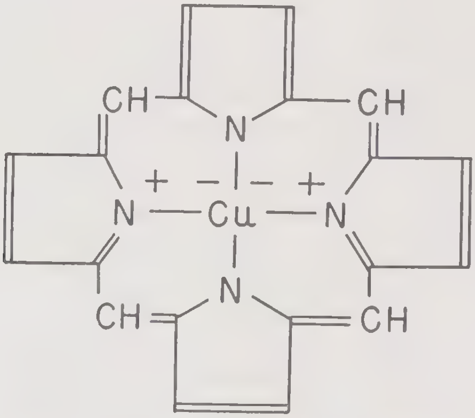

Fig. 3. A Copper Porphyrin Complex.

Proteins and other organic molecules to be found in plant and animal juices have innumerable possibilities for the formation of chelation compounds of great strength. By combining the effects of favorable or unfavorable bond angles, favorable or unfavorable atomic polarities, favorable or unfavorable reaction solvents, greater or lesser degrees of coordination, and greater or lesser amounts of chelation, any desired delicacy of balance between opposing forces may be achieved for utilization by biochemical systems. This interplay of forces gives richness and variety to the functions which can be achieved by any given chemical element and is undoubtedly responsible for the ever-increasing number of uses which we are finding for elements such as copper in metabolic processes.

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DISCUSSION

DR. THOMPSON: One of the things that I have noticed in working with the polarographic aspects of copper complexes is that two distinct waves are obtained from certain complexes in solution. The well-known case is that of copper in ammonia-ammonium chloride where the waves are about —0.225 and —0.500 volts. We have shown that with these copper-protein complexes we obtain one wave at about —0.250 to —0.260, showing a large shift to the negative. The second wave, which should have occurred earlier, comes at about —1.200. This, I believe, would be an excellent example of the complexing effect of these proteins, would it not? Incidentally, the effect seems to be independent of pH when working with protein complexes within reasonable limits.

DR. CORWIN: The complexes formed by proteins are usually much tighter than any water complexes. Aren't your two waves the cupric-cuprous wave and the cuprous-copper metal wave?

DR. THOMPSON: That is right.

DR. CORWIN: When there is only one wave, this represents the reduction of cupric directly to copper metal. When certain complexing groups are put around the copper, they will stabilize cuprous with respect to cupric, thus making the cupric-cuprous wave appear. With different groupings and different degrees of stability due to chelation and bond angle effects all sorts of interrelationships are possible. Cupric can be stabilized with respect to cuprous, or cuprous with respect to cupric and either or both with respect to hydrogen.

Dr. THOMPSON: That is demonstrated simply by shifting electrolytes in a copper solution when you have a single wave in the presence of chloride and a double wave with the ammonia complex.

Dr. CHANCE: What are the numbers of unpaired electrons that are possible with the cuprous and cupric states in solution?

Dr. CORWIN: In the cuprous state there are none unpaired, and in the cupric there is one. However, you may have a situation in which there is ionic bonding. If you had an ionic cupric chelate, you might have more than one unpaired electron. There should be a maximum of five in the cupric case. It should be possible to determine this by the magnetic susceptibility.

Dr. CHANCE: Are there cases known in which the magnetic data indicate five unpaired electrons for a cupric complex?

Dr. CORWIN: I have not made an exhaustive search of the magnetic data with respect to copper complexes. In addition, I am not quite confident of the interpretation of the magnetic data for complicated organic compounds.

Dr. CHANCE: Are the magnetic data complicated or is it just that copper compounds are difficult?

Dr. CORWIN: Iron compounds have been much more investigated than copper. A most interesting case is that of hemoglobin. This appears to have five unpaired electrons, while oxyhemoglobin has none. With hemoglobin there is a large paramagnetism, oxygen too is paramagnetic, and yet when the two combine to form oxyhemoglobin the paramagnetism disappears. There might be similar cases with copper-protein complexes.

Dr. CHANCE: Do cases of two or three unpaired electrons occur in copper compounds?

Dr. CORWIN: I am not aware of such cases, but I have not made a search with this question in mind.

Dr. COLEMAN: Dr. Corwin, in the light of your discussion, what sort of complexes would you expect between copper and the sulfur-containing amino acids?

Dr. CORWIN: We should expect strong complexes. There would be a number of possibilities depending upon the relative positions of atoms of sulfur, nitrogen, and oxygen available for complex formation with the copper. There is the possibility of a sulfur-copper-nitrogen complex, which should be stronger than a sulfur-copper-oxygen complex, which in turn would be stronger than the usual nitrogen-copper-oxygen complex. I may suggest that it might be possible to obtain structural information about the groups surrounding copper, since complexes with sulfur-containing amino acids should be appreciably stronger than those with non-sulfur acids.

Dr. DAWSON: Does the fact that the copper in some of the copper oxidases is removable by dilute acid or by cyanide give you a clue as to what should not be considered as complexing groups?

Dr. CORWIN: Yes, to a certain extent. You can make a stability series. Amino acids will break the copper-ammonia complexes and take the copper. The copper-ammonia complex is part of a dynamic system. If you put in acid and neutralize the ammonia you break the complex. This is because free ammonia is the species that reacts with copper. Ammonium ion does not. If you make more and more ammonium ions with acid, you shift the equilibrium and break the complex. Because of the fact that amino acids make tighter complexes than ammonia, you would have to have stronger acid to break the amino acid complex than to break the ammonia complex. The oxygen bonds of water complexes are relatively easily broken by amino acids, single nitrogen bonds are relatively easily broken, sulfur bonds are harder to break, doubly chelated nitrogen bonds are still harder to break. In general, amino acids should break singly bound nitrogen or doubly bound chelated oxygen bonds.

Dr. DAWSON: Possibly the latter case might be that in the oxidase.

Dr. CORWIN: It might be.

THE COPPER PROTEIN, ASCORBIC ACID OXIDASE

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INTRODUCTION

It has long been recognized that the element copper plays an important role in the respiration of lower forms of animal life. The development of interest and research on the hemocyanins, the copper-containing respiratory proteins found in the blood of certain marine animals, started about one hundred years ago. However, until two or three decades ago there was little interest in the biological function or significance of copper in plants and higher animals. Early workers were inclined to believe that the small amounts of copper found in plant and animal tissues were of accidental origin. It was not until about 1921 that copper was accepted as a definite constituent of all such tissues (3).

During the past two decades there has come about a realization that trace amounts of copper are essential to the biological synthesis of hemoglobin and the structurally related iron porphyrin-protein enzymes, i. e., the cytochromes, cytochrome oxidase, peroxidase, catalase, etc. Trace amounts of copper have likewise been shown to be essential for the development of the plant pigment, chlorophyll. A number of biological catalysts, enzymes, have been found to contain copper as an integral and essential part of their structure. In addition, a number of copper protein complexes of as yet unknown biological function have been isolated since 1938 (Table 1). These findings have initiated a marked interest in the nutritional and functional significance of trace amounts of copper.

Questions Concerning the Copper-to-Protein Bond

It is important to note that practically nothing is known at the present time in regard to the nature of the chemical bond or linkage between the copper and the protein in the copper proteins tabulated above. Only in the case of one of the enzymes, ascorbic acid oxidase, is there some recent information in regard to the nature of the copper-protein bond. In fact, only in the case of the hemocyanins and two of the enzymes, tyrosinase and ascorbic acid oxidase, is there any information at all concerning the size, shape, and electrical properties of such protein molecules. No information is at hand concerning the chemical groupings that must be available in such proteins for bonding with the metal.

TABLE 1

COPPER PROTEINS*

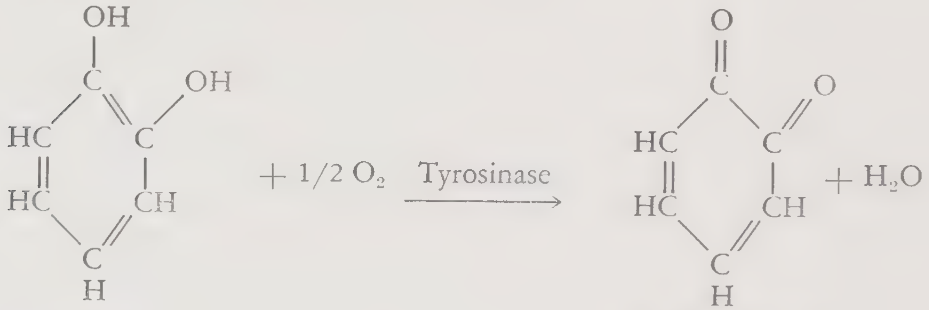
Name	Biological Function	Principal Source	Per cent Cu
Hemocyanins	Respiratory Pigments	Numerous marine animals	0.15—0.26
Polyphenol Oxidase	Enzyme	Plants; potato and mushrooms	0.30
Tyrosinase	Enzyme	Plants; potato and mushrooms	0.25
Laccase	Enzyme	Plants; various Lac trees and mushrooms	0.24
Catechol Oxidase	Enzyme	Plants; sweet potato	0.20
Ascorbic Acid Oxidase	Enzyme	Plants; crook-neck squash and cucumber	0.26
Hepatocuprein	Unknown	Liver; ox	0.34
Hemocuprein	Unknown	Blood; ox, sheep, and horse	0.34
Copper Protein	Unknown	Milk; cow	0.19
Copper Protein granules	Unknown	Yeast cells	0.12

*Data for the table taken from "The Copper Proteins," by C. R. Dawson and M. F. Mallette, *Advances in Protein Chemistry* 2, 179-248. (1945).

Until some information, even though only qualitative, is available concerning the relative strength of the copper to protein bond and the nature of the chemical groupings involved, there can be no satisfactory answer to a number of intriguing and important questions concerning the biological function and significance of this metal.

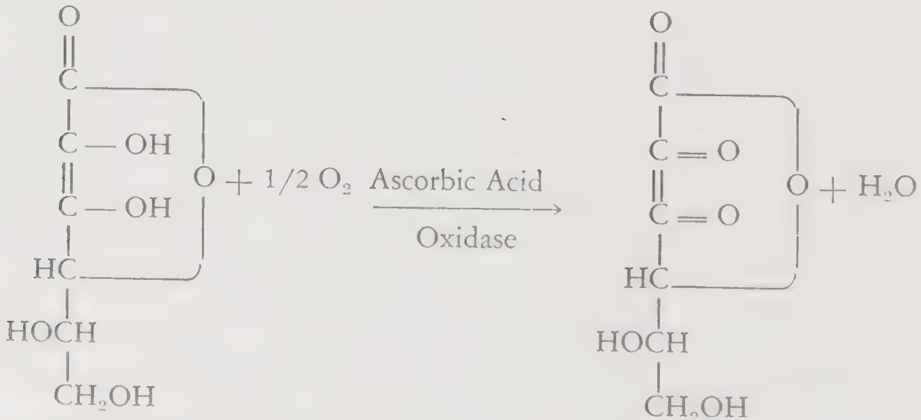
For example: Why is it that copper is not found randomly distributed in chemical combination with the many different proteins of plant and animal tissues and body fluids? Actually, the metal is found to be concentrated within a limited area or organ of the living body and is firmly bound to a specific protein or type of protein. From other proteins the copper may be removed by simple dialysis without injury to the protein. From these specific proteins, the metal is much more difficult to remove, and such removal results in loss of biological function and frequent injury to the protein. What is the structural feature of the specific protein that makes it so different from other proteins in regard to the chemical binding of copper?

Another intriguing question concerns the remarkable biological specificity exhibited by the copper protein enzymes. For example, ascorbic acid oxidase and tyrosinase are two copper proteins that cannot be differentiated on the basis of their copper content and protein properties. Yet they can be easily differentiated on the basis of their specific enzyme activity. One catalyzes the aerobic oxidation of ascorbic acid (Vitamin C) and has no effect whatsoever on the oxidation of phenols such as catechol. The other oxidase (tyrosinase) catalyzes the aerobic oxidation of catechol and certain structurally related phenols but has no action on ascorbic acid. Actually, ascorbic acid and catechol have a structural feature in common (an ene-diol grouping) and it is this grouping which is involved in the oxidation in both cases.



Catechol

o-Benzoquinone



L-Ascorbic Acid

Dehydroascorbic Acid

What is the chemical difference betwen these two proteins that enables them to display such a high order of specificity of function? Is there a difference in the nature and strength of the copper-protein bonds involved?

All of the copper enzymes listed in the table exhibit a common property when purified specimens of the oxidases are compared. That is, they all undergo a marked inactivation during the reaction which they are catalyzing. The oxygen-uptake curves shown in Fig. 1 illustrate the phenomenon, and are typical of those obtained when a purified sample of any copper oxidase is employed. When a relatively small amount of the enzyme is employed, the rate of reaction rapidly and continuously decreases until the enzyme is

Figure I

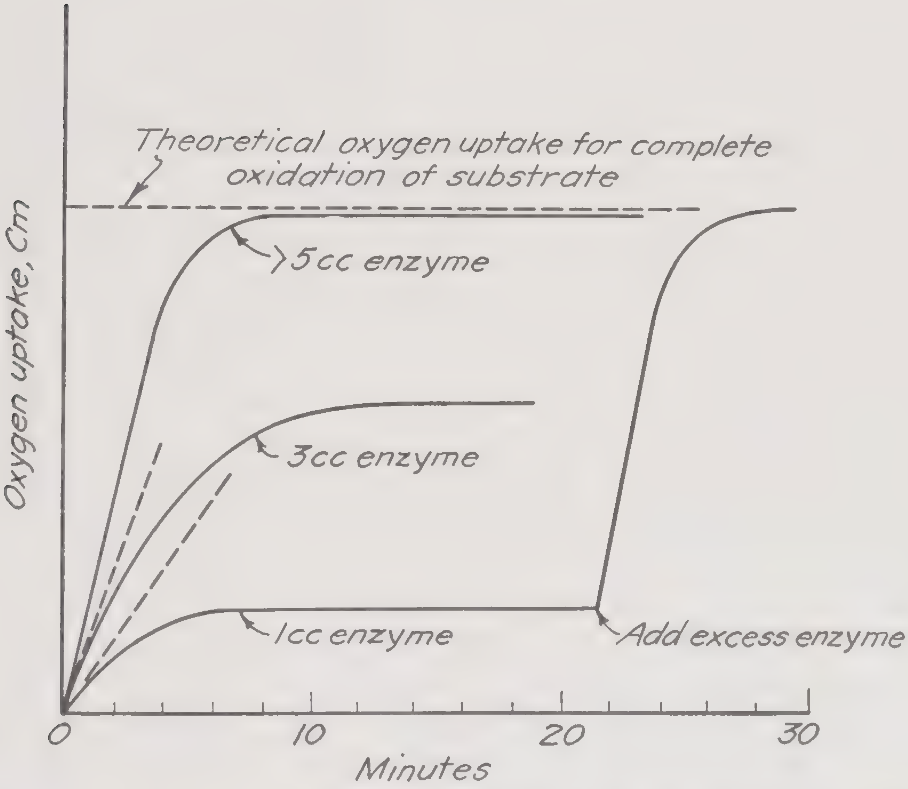


Fig. 1. Oxygen uptake of purified copper oxidase for different amounts of enzyme.

completely inactivated. The addition of more enzyme at this point results in further oxidation of the substrate. Only when a relatively large amount of the enzyme is employed does a complete oxidation of the substrate occur. This reaction inactivation of the copper oxidases is irreversible and is not due to any products of the reaction known to be formed.

The fact that rapid reaction inactivation is a property characteristic of all of the purified copper oxidases so far studied leads to the thought that the enzymes may irreversibly lose their copper during the catalytic process and thereby become inactive. In other words, the inactivation may be due to an irreversible rupture of the copper-protein bond. Only in the case of ascorbic acid oxidase is there recent information concerning this question.

Many questions other than those raised above must be answered before a complete understanding of the nutritional and biological significance of copper can be had. The above questions, however, serve to illustrate the importance of seeking information concerning the nature of the chemical bond between the copper and certain specific proteins.

The copper oxidases are well suited for such studies, for they possess a powerful biological (enzymatic) activity which is dependent on the presence of both copper and a specific protein. The activity is easily measured in a quantitative fashion, and such measurements serve as guides during chemical and physical investigations of such copper-protein complexes.

Only relatively recently has it been possible to prepare significant amounts of certain of these copper oxidases in a state of purity sufficient for chemical and physical investigation. The effort and time that go into preparing one of these enzymes in pure form are often comparable to those involved in isolating a rare element. The enzyme is frequently unstable at one or more stages in the purification procedure, and great losses in activity are encountered throughout the process. The yield of pure enzyme is frequently of the order of 1 mg. per 40 to 50 lbs. of starting material. During the past fifteen years, methods for obtaining the copper-protein enzymes in a high state of purity, and techniques for measuring the activity of such enzymes, have been under continuous investigation in the laboratories of the Chemistry Department of Columbia University. Much of the work has been concerned with the two copper oxidases, tyrosinase and ascorbic acid oxidase. The recent studies on highly purified ascorbic acid oxidase, involving ion-exchange columns and radioactive copper, have provided some very interesting information in regard to some of the questions posed earlier.

Ascorbic Acid Oxidase

In 1930, Szent-Györgyi (34, 35) reported that cabbage leaves contain an enzyme "hexoxidase" which catalyzes the direct aerobic oxidation of "hexuronic acid." When shortly thereafter "hexuronic acid" was identified by King and coworkers (14) as L-ascorbic acid, the antiscorbutic vitamin, many workers became interested in its biological oxidation. Since that time, numerous investigators, working with a wide variety of plant extracts, have described the preparation and properties of ascorbic acid oxidase solutions (6, 7, 19, 21, 23, 24, 27, 28, 31, 37, 39).

Role of Copper. Interest in the role of copper in the biological oxidation of ascorbic acid was initiated in 1921 by Hess and Unger (9), who observed that the stability of the antiscorbutic vitamin in aqueous aerobic systems was markedly decreased by the presence of copper salts. In 1936, Barron, DeMeio, and Klemperer (2) confirmed and extended these observations, using crystalline ascorbic acid. In the following year Stotz, Harrer, and King (32) reported that the catalytic activity of several plant juices in decomposing ascorbic acid was indeed dependent on their copper contents, and such activity was lost upon treatment with complexing agents such as cyanide, sulfide, diethyldithiocarbamate, 8-hydroxyquinoline, etc., which were known to form stable complexes with copper.

Within a few years it was observed that the oxidase activity of several purified ascorbic acid oxidase preparations paralleled their copper content (19, 21, 23, 27, 28, 31), and many workers then looked upon the activity as that of a new copper protein oxidase. However, the fact that cupric ions, and cupric ions together with inert protein, also catalyze the aerobic oxidation of ascorbic acid in aqueous solutions led other workers to express reservations and doubt concerning the existence of a specific copper-protein enzyme, ascorbic acid oxidase.

Reservations Concerning the Copper-Protein Nature. Among the first to question the existence of ascorbic acid oxidase were Barron, Barron, and Klemperer (1), who suggested in 1936 that the oxidation of the vitamin in plant juices and other biological fluids could be attributed solely to the catalytic activity of ionic copper and hemochromogen catalysts. A few years later, McCarthy, Green, and King (22) demonstrated that synthetic mixtures of copper ion and inert protein (egg albumin) exhibited many of the characteristic properties of an enzyme, such as loss of activity on heating, pH effects, and the effect of copper inhibitors. Because of these similarities, they suggested that the evidence at that time for the existence of a specific copper-protein possessing ascorbic acid oxidase activity was rather tenuous. More recently, this view has been reemphasized by the publications of Lampitt and Clayson (16, 17) and Mommaerts (25).

Lampitt and Clayson have observed that calcium phosphate gels containing a trace of ionic copper possess the ability to catalyze the aerobic oxidation of ascorbic acid (18), and such catalytic activity is destroyed or considerably reduced by heat. They have argued the possibility that such a synthetic " ascorbic acid oxidase," with or without combined protein, is identical with that occurring naturally. They have assumed that the catalytic activity of " ascorbic acid oxidase " may be attributed to traces of ionic copper resulting from a dissociation reaction of the following type:

$$\mathrm{Cu(P)} \rightleftarrows \mathrm{Cu}^{++} + \mathrm{(P)}^{=}$$

where P designates non-specific protein matter or other colloidal material such as calcium phosphate gel. On this premise they have questioned the function of the protein moiety and have criticized the work of previous investigators as failing to distinguish between Cu^{++} and copper-protein.

Evidence for the Specific Copper Protein View. At the present time there exists strong experimental evidence in opposition to the view that ascorbic acid oxidase activity may be attributed solely to ionic copper in loose combination with non-specific protein matter or other colloidal material. Such systems do indeed possess the ability to catalyze the aerobic oxidation of ascorbic acid in aqueous solutions, but it is a feeble pseudo-activity in comparison to that of purified ascorbic acid oxidase preparations. The evidence strongly supports the view that such purified ascorbic acid oxidase preparations contain a copper-protein complex or conjugate involving a specific protein. Under physiological conditions, the copper is firmly bound to the protein and does not dissociate to yield ionic copper.

Much of the evidence in support of the view that ascorbic acid oxidase is another example of a physiologically active and specific copper-protein has been available for several years. Other points of evidence are very recent. Before considering the recent findings it seems advisable to review briefly some of the earlier observations.

As previously pointed out, several investigators (19, 21, 23, 27, 28, 31) have reported that throughout successive stages in the purification of a heat-labile, non-dialyzable factor, containing copper and possessing ascorbic acid oxidase activity, the oxidase activity parallels the non-dialyzable (bound) copper content. The methods of purification used by these investigators were those commonly employed for protein fractionations, and the active principle of the preparation described by Powers, Lewis, and Dawson (27) was a bluish-green globulin containing 0.24 per cent copper. Although this preparation was more than one and one-half times as active, on a dry weight basis, as that described by Lovett-Janison and Nelson and which contained 0.15 per cent copper (19), the enzyme activity per unit of copper was found to be the same in both cases (Table 2).

Lovett-Janison and Nelson (19) emphasized that the catalytic activity of their oxidase preparations per unit of copper was more than one thousand times that observed in ascorbic acid systems containing only ionic copper in equivalent amount. Similarly, Straub (33) demonstrated that the catalytic activity of cucumber juice after ashing was only a very small fraction of that possessed by the juice before ashing. Meiklejohn and Stewart (23) found that the copper of the enzyme could be removed by dialysis against cyanide solution. The resulting inactive enzyme solution was reactivated by the addition of excess copper sulfate. The restored activity was greater than that of the amount of copper sulfate used for the restoration.

One of the most significant points that has apparently been overlooked by Lampitt and Clayson, and others, is the fact that ascorbic acid oxidase preparations exhibit a substrate specificity that cannot be explained in terms of cupric ion catalysis. Under suitable conditions the cupric ion catalyzes the aerobic oxidation of a wide variety of substances, including various phenols, cysteine, phenylene diamines, dihydroxymaleic acid, and numerous compounds structurally related to ascorbic acid (5). Ascorbic acid oxidase, on the other hand, has no action on phenols, cysteine, phenylene diamines, etc., and exhibits a marked specificity dependent on the structural features of the ascorbic acid molecule and its derivatives (5). L-Ascorbic acid and its derivatives are oxidized much more rapidly by the enzyme than the respective enantiomorphs (11).

TABLE 2

COMPARISON OF SEVERAL ASCORBIC ACID OXIDASE PREPARATIONS
(Powers, Lewis, and Dawson, *J. gen. Physiol.*, 27; 167. 1944)

Preparation No.	Concentration units/cc.	Units/γCu	Per cent Cu	Units/mg. dry weight
14–3A1	594	456	0.11	510
14–IIB3B2–A	675	407	0.15	592
14–IIB3C2	600	400	0.15	588
11–L.J.*	531	406	0.15	612
12–L.J.*	516	490	0.15	631
15–OI	1970	450	0.24	1060
		Av. . . . . 428 ± 25†		
15–OI‡	3125	715	0.24	1680

* Best preparations obtained by Lovett-Janison and Nelson.

† Lovett-Janison and Nelson preparations and the modified value (715) are not included in the calculation of the average.

‡ Activity measured after modified dilution procedure.

Another significant point that has been known for some time is the fact that the aerobic oxidation of ascorbic acid as catalyzed by purified ascorbic acid oxidase preparations results in an oxygen consumption of one gram atom per mole of ascorbic acid without hydrogen peroxide formation (28, 29). Cupric ion catalysis, on the other hand results in greater oxygen uptake and production of hydrogen peroxide (4, 8, 20, 29, 30; Fig. 2).

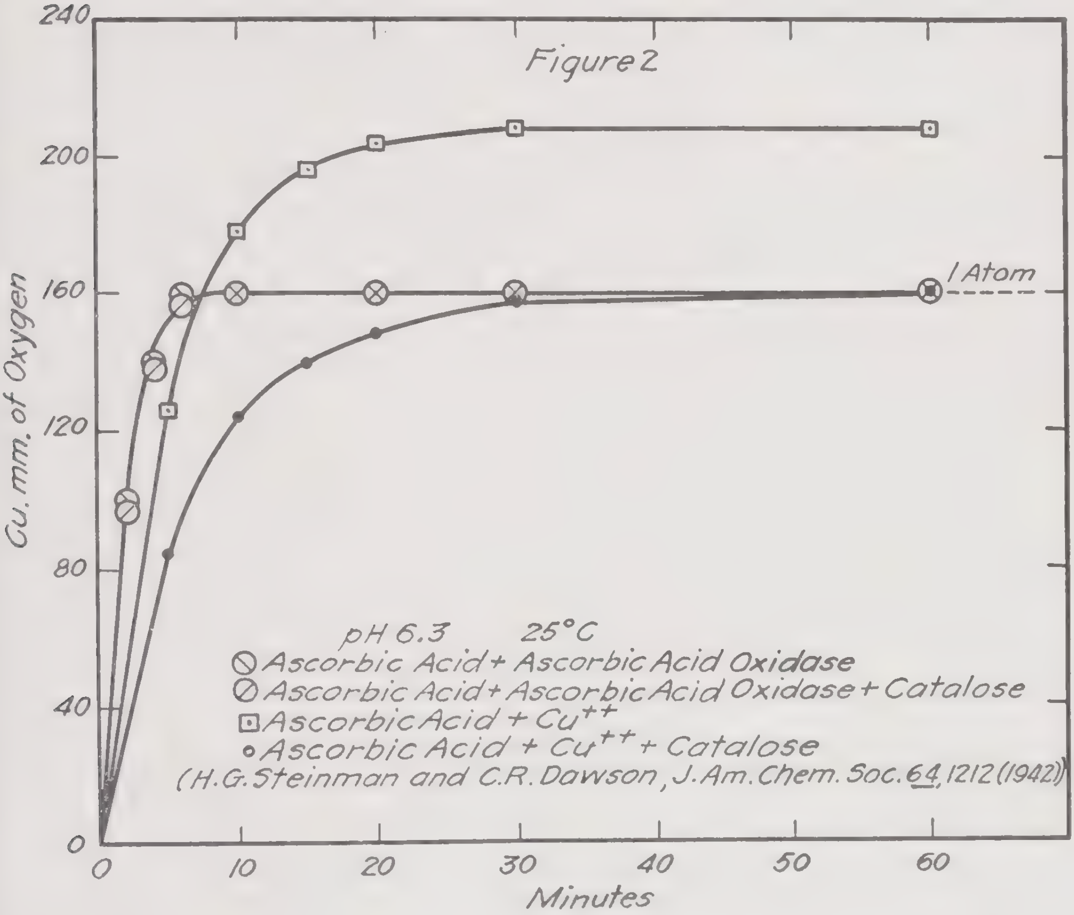


Fig. 2. Aerobic oxidation of ascorbic acid by ascorbic acid oxidase compared with that by cupric ion, with and without added catalase.

Recent Studies

Homogeneity, Molecular Weight, and Color. Recently several ascorbic acid oxidase preparations of varying levels of purity, activity, and copper content have been prepared in the Columbia

Laboratories from the summer crook-neck squash (*C. pepo condensa*) and have been examined electrophoretically and in the ultracentrifuge (6). The results of this investigation strongly support the view that ascorbic acid oxidase is a specific copper-protein.

Under the same conditions of buffer salt, pH, etc., the electrophoretic mobility of the active component of each of several preparations was found to be the same, even though the preparations contained different amounts of extraneous protein matter and accordingly had different specific activities and copper contents. Typical results are shown in Table 3 and Fig. 3. By extrapolating the curve shown in Fig. 3, it can be estimated that the isoelectric point of ascorbic acid oxidase, i. e., the pH of zero mobility, is in the region of pH 5-5.5. The enzyme exhibits an optimum activity at pH 5.6 (27; Fig. 4). It would appear, therefore, that the enzyme is most active under conditions where it carries a small negative charge. The exact pH of the isoelectric point cannot be conveniently determined electrophoretically because the copper of such copper proteins becomes ionic in character in solutions buffered below values

TABLE 3

Typical Electrophoresis Data

(F. J. Dunn and C. R. Dawson, *J. biol. Chem.*, In Press)

Prep. No.	% Cu	Specific activity (units/mg.)	pH of exp.	Mobility of active component	Number of components	% of active component (Est.)
2	0.22	1530	5.75 6.13 6.95 7.74	0.57 1.05 1.83 2.40 ($\times 10^{-5}$)	2 3 2 2	70 70 70 ..
6A	0.26	1925	5.75 7.38	0.62 2.26 ($\times 10^{-5}$)	1 1	100 100
6B	0.24	1850	6.38 6.76 7.38 7.90	1.24 1.52 2.22 2.49 ($\times 10^{-5}$)	2 2 2 2	90 90 95 90

Note: In all cases, the buffer was KH_2PO_4—KOH of 0.2 ionic strength.

of about 5. This transformation of the enzyme copper to a dialyzable ionic form is accompanied by loss in enzymatic activity.

Analogous results in regard to the nature of the enzyme were obtained in the ultracentrifuge. The degree of homogeneity of each of the several preparations, as estimated from the scanning photographs taken during the sedimentation, agreed with that estimated from the electrophoresis diagrams, and the sedimentation constant of the active component of each preparation was found to be the same (6.9 Svedberg units, corrected to 20°C.).

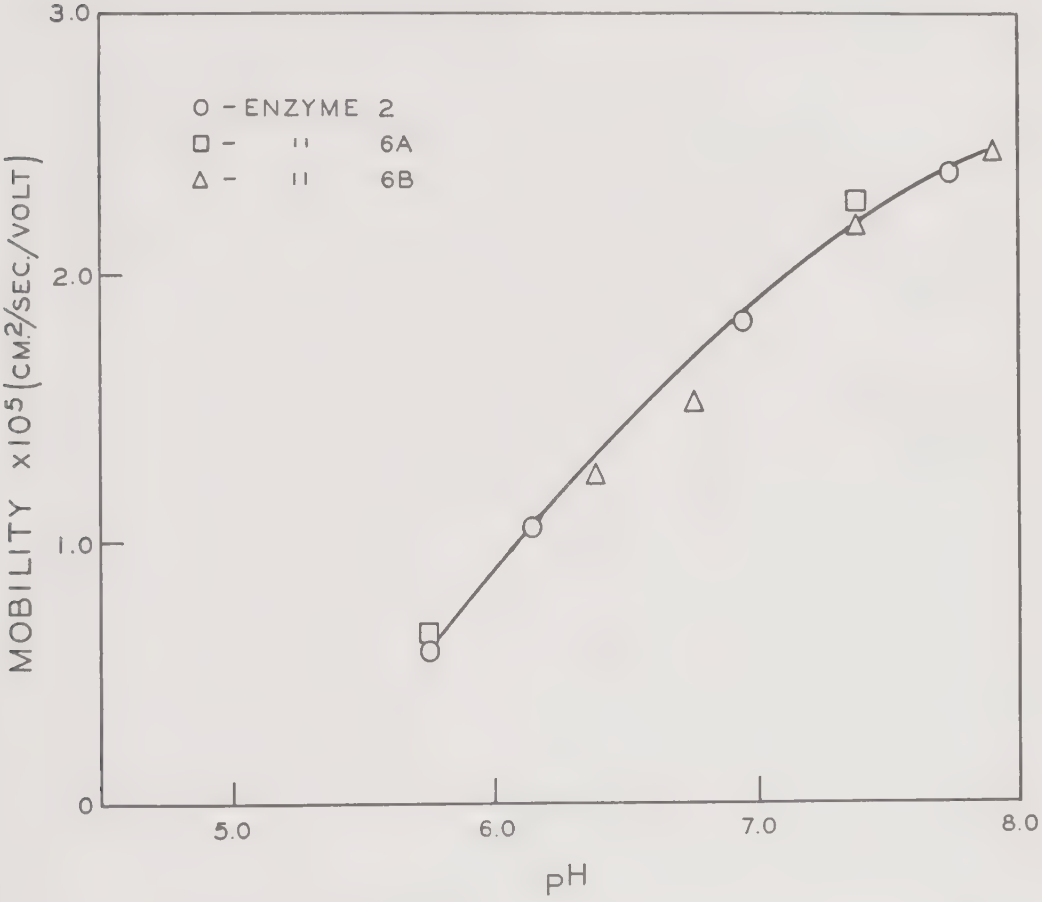


Fig. 3. The variation in mobility with pH of the active component of three ascorbic acid oxidase preparations.

By modifying the former procedure of preparation so as to include a step involving differential ultracentrifugation and fractional precipitation by dialysis, an enzyme was obtained having a specific activity of nearly 2000 units/mg. dry weight, and containing 0.26 per cent copper. This specific activity and copper content is significantly higher than that of any ascorbic acid oxidase previously described. However, the activity per gamma of copper (740 units) is about the same as that reported by previous workers. The enzyme, which was distinctly blue-green in color, was found to be homogeneous as judged by electrophoresis and ultracentrifuge criteria applied over a range of pH. The sedimentation and diffusion constants indicated a molecular weight of about 150,000, which corresponds to six copper atoms per molecule.

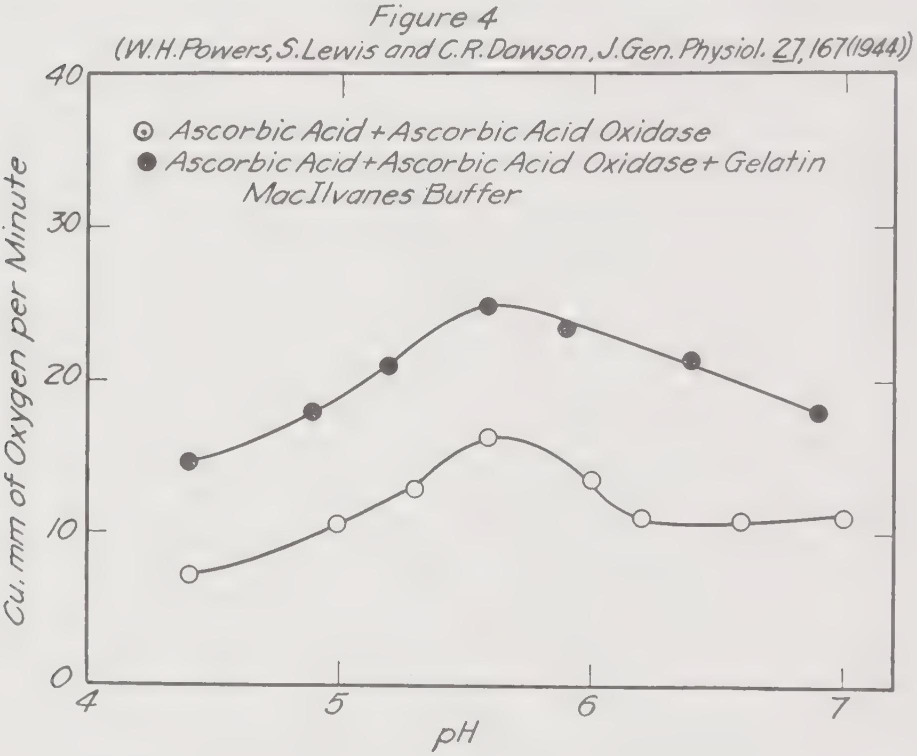


Fig. 4. Activity of ascorbic acid oxidase at various pH values.

The blue-green color of the purified enzyme was observed to be dependent on the presence of oxygen. When the concentrated enzyme solution was sealed in tubes which had been evacuated and then filled with nitrogen, the color slowly changed to a yellow or yellow brown. As soon as the contents were exposed to air, the blue-green color rapidly reappeared. The same kind of reversible color change was also observed when ascorbic acid solution was added dropwise to the concentrated blue-green enzyme in the tube, except that the decolorization was much more rapid. Upon shaking the contents of the tube in air, the blue-green color rapidly reappeared. Such experiments were repeated many times with the same enzyme solution. Before attempting to discuss the implications of the blue-green to yellow color transformation, it will be helpful to review briefly some of the information that has been obtained recently as the result of studies on the enzyme with an ion-exchange resin (12) and with radioactive copper (13).

Ascorbic Acid Oxidase and Radioactive Copper. In contemplating the use of a radioactive isotope of copper as a tool for gaining more information about the nature of the copper to protein bond in ascorbic acid oxidase, it was desired to expose the enzyme in solution to a high concentration of radioactive cupric ions, and after a suitable period, quantitatively to separate the active enzyme from the extraneous copper. If the copper to protein bond in the enzyme were dissociable, as postulated by Lampitt et al. (18), one would anticipate an introduction of radioactive copper into the enzyme via an exchange reaction of the following type, in which the asterisk is used to denote radioactivity:

$$\mathrm{Cu\,P} + \overset{*}{\mathrm{Cu}}{}^{++} \rightleftarrows \overset{*}{\mathrm{Cu}}\,\mathrm{P} + \mathrm{Cu}^{++}$$

It was apparent that three special requirements had to be met:

1) The radioactive copper must not by its own emanations cause any damage to the activity of the enzyme.

2) Because of the relatively short half-life of radioactive copper (Cu^{64}, 12.8 hrs. (41)), the entire exchange study had to be designed for completion in a short time.

3) A rapid and quantitative method for separating the enzyme from extraneous ionic copper had to be developed.

The first requirement presented no difficulties, for in no case was any damage to the activity of the enzyme observed as the result of contact with radioactive Cu^{64} (emitting B^- and B^+ particles of 0.58 mev. and 0.66 mev. (40), respectively). The last two requirements posed a problem in technique which was solved by the use of an ion exchange resin, Amberlite IR-100, AG. The usual methods of removing extraneous metallic ions from proteins proved not to be satisfactory. A simple dialysis process was too slow, and both electrodialysis and protein precipitation techniques resulted in an unsatisfactory recovery of the enzyme activity. A diagram of the ion-exchange column is shown in Fig. 5. The column and reaction chamber were designed so that the exchange experiment and separation could be carried out either under aerobic or anaerobic conditions.

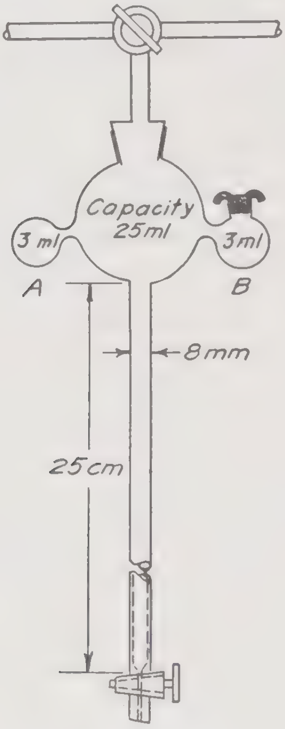


Fig. 5. Diagram of the ion exchange column used for removing ionic copper from ascorbic acid oxidase preparations.

All of the experiments were run at room temperature in solutions buffered to pH 5.6 with 0.1 M acetate buffer. This pH was selected because it is close to that at which the enzyme is found naturally, and it is also the pH of its optimum activity. Furthermore, at this pH the enzyme exists as an anion and should not be adsorbed onto a cation exchange resin.

After establishing that the purified enzyme would pass through the column without any loss in its copper content or activity, the efficiency of the column in removing large amounts of added ionic copper was tested. The results with several different ascorbic acid oxidase preparations are shown in Table 4. In all experiments the rate of flow through the column was controlled to about 1 drop per 30 seconds, so that about 2 hours was required to collect 10 ml. of effluent. At this rate of flow the capacity of the column was sufficient for the complete removal of more than ten times the amount of copper used in any of the experiments. The results in Table 4 show that exposure of the enzyme to relatively large amounts of added Cu^{++} ion has no harmful effect on the enzyme activity, and the additional Cu^{++} ion can be quantitatively removed by a cation exchange resin.

TABLE 4

Removal of Extraneous Ionic Copper from Ascorbic Acid Oxidase Solutions

The activities and copper contents listed below are those of the enzyme solutions before adding extraneous cupric ions and after passage of the mixtures through the ion exchange column.

(M. Joselow and C. R. Dawson, *J. biol. Chem.*, In Press)

Enzyme No.	Before		Amt. of Cu^{++} added γ	Time of contact before passage hrs.	After	
	Activity units/ml.	Cu content γ/ml.			Activity units/ml.	Cu content γ/ml.
*	570	2.0	20	1	580	2.1
1	850	2.6	26	20	875	2.5
2	1780	3.6	36	1	1820	3.5
3	3040	4.5	4.5	1	3070	4.5

* An uncharacterized sample of enzyme that was not used for further work.

The results obtained when the enzyme was mixed with a solution of radioactive cupric ions and then passed through the column are shown in Table 5. It is to be noted that in no case was there a significant amount of radioactivity found in the enzyme effluent from the column. Furthermore, the enzyme came through with its full activity and original copper content. In other words, during a period of as long as 16 hours of contact between the enzyme and the radioactive cupric ions, no exchange between the copper of the enzyme and the radioactive cupric ions of the solution occurred. These data clearly establish that in the case of the "resting" or non-functioning enzyme there is no dissociation of the copper from the ascorbic acid oxidase protein.

TABLE 5

Ascorbic Acid Oxidase and Cu^{64}

The activities and copper contents listed below are those of the enzyme preparations before adding extraneous radioactive cupric ions and after passage of the mixtures through the resin columns.

(M. Joselow and C. R. Dawson, *J. biol. Chem.*, In Press)

Enzyme No.	Before		Amt. of Cu^{64} added (in 1 ml. .1 M acet. buffer) γ	Time of contact before passage through column hrs.	After		Radioactivity		Exchange %
	Cu content γ/ml.	Oxidase activity units/ml.			Cu content γ/ml.	Oxidase activity units/ml.	Enzyme effluent cts/min	Comp. std.* cts/min	
1 (2 ml.)	2.6	850	5.2	16	2.5	840	0†	315±9	0
2 (1 ml.)	3.6	1780	3.6	1	3.6	1730	2±2	684±12	0
4 (1 ml.)	6.4	4530	6.4	1	6.5	4670	0†	450±7	0

* The differences in the specific activity of the standards are due to the fact that these experiments were all done on different occasions with different preparations of radioactive copper.

† Observed count was less than standard error of count (±2).

The Copper of the Reaction-Inactivated Enzyme. As previously pointed out, one of the most striking features of the aerobic oxidation of ascorbic acid as catalyzed by the enzyme ascorbic acid oxidase is the simultaneous rapid inactivation of the enzyme. This reaction inactivation has led to the thought that the enzyme may irreversibly lose its copper during the catalytic process, and thereby become inactive. For this reason, it was essential to determine the fate of the copper of the reaction-inactivated ascorbic acid oxidase before undertaking any exchange experiments between the enzyme and radiocopper in the presence of substrate. It was also necessary to know whether contact of the substrate itself with the enzyme, without a reaction occurring (in an inert atmosphere), would sever the linkage between the copper and the protein, possibly by changing the valence state of the copper.

The reaction inactivation experiments were performed in the following way. The buffered enzyme and the buffered ascorbic acid solution were placed in the side arms of the resin column reaction chamber (Fig. 5) and the system was evacuated. Oxygen was then admitted, and the enzyme and substrate were mixed and agitated for five minutes. The system was then evacuated and again agitated with oxygen for five minutes. This process was repeated five times during the period of an hour, and then the mixture was run through the column.

In order to study the ascorbic acid-ascorbic acid oxidase system under an inert atmosphere (no oxidation reaction), the enzyme and substrate solution (previously bubbled with nitrogen) were placed in the side arms and the system was evacuated as before. Nitrogen was then admitted and the enzyme and substrate were mixed. After the mixture was subjected to a series of evacuations and fillings with nitrogen in a manner similar to the oxygen experiment, it was allowed to pass through the resin column.

The results of these experiments are given in Table 6. They reveal that in the case where a reaction between the enzyme and substrate was permitted (oxygen atmosphere), extensive inactivation of the enzyme occurred, but there was no loss in copper to the column. In other words, the reaction inactivation of this particular copper oxidase does not appear to be due to a rupture of the copper-to-protein bond. The experiment under a nitrogen atmosphere likewise indicates that ascorbic acid itself does not rupture the copper-to-protein bond, i. e., if ascorbic acid reduces the copper of the enzyme from the divalent to the monovalent state, the reduced valence state is also firmly bound to the protein.

The same results and conclusions, in regard to the retention of copper by the reaction-inactivated enzyme, were also obtained by a dialysis experiment in which the enzyme-substrate reaction was allowed to proceed slowly during the simultaneous dialysis of the system (12).

Ascorbic Acid Oxidase, Ascorbic Acid, and Cu^{64}. To determine whether or not the copper of the functioning enzyme would exchange with radioactive cupric ions, the substrate, ascorbic acid, was added to the mixture of buffered enzyme and Cu^{64} in the presence of oxygen. In this system the enzymatic oxidation of ascorbic acid occurred in the presence of radioactive cupric ions. After a suitable period (about 1 hour), the reaction mixture was passed through the resin column as previously described, and the effluent enzyme was examined for enzyme activity, copper content, and radioactivity. As a control, the same experiment was repeated under a nitogen atmosphere. In this case no enzymatic oxidation of the substrate occurred, and the effect of ascorbic acid itself on the exchange could be evaluated. The results of such experiments with three different enzyme preparations are shown in Table 7.

TABLE 6

RETENTION OF COPPER OF INACTIVATED ASCORBIC ACID OXIDASE

The activities and copper contents listed below are those of the enzyme before adding substrate and after passage of the mixture through the ion exchange column.

Atmosphere	Before		After	
	Activity units/ml.	Cu content γ/ml.	Activity units/ml.	Cu content γ/ml.
N_2	850	2.6	555*	2.5
O_2	850	2.6	24	2.6
O_2	850	2.6 $+26\gamma Cu^{++}$	8	2.5

* Since ascorbic acid oxidase rapidly catalyzes the oxidation of ascorbic acid on coming in contact with air after leaving the column, and in so doing becomes inactivated, this figure had to be obtained indirectly (see M. Joselow and C. R. Dawson, *J. biol. Chem.*, In Press).

It can be seen from the data in the table that in each case where the enzyme was performing its catalytic function (oxygen atmosphere), a significant exchange occurred between the copper of the enzyme and the radioactive cupric ions of the solution. This exchange (about 30 per cent) was accompanied in each case by a loss in oxidase activity (due to inactivation during the reaction), with no measurable change in the copper content of the enzyme.

TABLE 7

ASCORBIC ACID OXIDASE, ASCORBIC ACID, AND Cu^{64}

The activities and copper contents listed below are those of the enzymes before adding extraneous radiocopper and ascorbic acid, and after passage of the mixtures through the resin columns.

(M. Joselow and C. R. Dawson, *J. biol. Chem.*, In Press)

Enzyme No.	Atmosphere	Before		Amt. of ascorbic acid added (in 2 ml. M acet. buffer) mg.	Amt. of Cu^{64} added (in 1 ml. .1 M acet. buffer) γ	After		Radioactivity		Exchange %
		Cu content γ/ml.	Oxidase activity units/ml.			Cu content γ/ml.	Oxidase activity units/ml.	Enzyme effluent cts/min	Comp. std. cts/min	
1 (2 ml.)	O_2	2.6	850	50	5.2	2.5	23	273±7	1613±18	34±3
	N_2	2.6	850	50	5.2	2.5	555*	46±4	1613±18	6±1
2 (1 ml.)	O_2	3.6	1780	100	3.6	3.3	35	46±2	306±6	30±2
	O_2†							(9±2)	(56±3)	(32±6)
	N_2	3.6	1780	100	3.6	3.7	1160*	8±2	684±12	2±1
4 (1 ml.)	O_2	6.4	4530	100	6.4	6.3	285	14±1	100±3	27±2
	N_2	6.4	4530	100	6.4	6.3	2950*	1±1	100±3	3±1

* These values could be obtained only indirectly (see footnote to Table 6).

† An aliquot of the effluent from the experiment under oxygen was repassed through another fresh resin column of the same length as the original. Counts on the effluent from the repassage are recorded in parentheses. The fact that the percentage exchange (last column) did not change significantly confirms the non-dissociable nature of the exchanged copper.

In all cases where a nitrogen atmosphere was employed to prevent enzymatic function, no significant exchange was observed. In other words, the ascorbic acid *per se* did not cause the exchange.

Component of the Reaction Mixture and Cu^{64}. The principal substances present in a reaction mixture in which the enzymatic oxidation of ascorbic acid is occurring are ascorbic acid and its oxidation product, dehydroascorbic acid, the active enzyme and the inactivated enzyme. As previously pointed out, the active enzyme by itself does not undergo exchange with radioactive copper. In order to determine whether or not any of the other components of the reaction mixture might be responsible for the observed exchange, each of these substances and mixtures of them were subjected to exposure to, and separation from, radioactive cupric ions under conditions similar to those described above. In no case was any significant radioactivity found in the effluent from the resin column.

On the basis of such experiments it may be concluded that the exchange observed during the reaction and inactivation of ascorbic acid oxidase cannot be attributed to any of the following:

1) ascorbic acid,
2) dehydroascorbic acid (or its decomposition products),
3) inactivated ascorbic acid oxidase,
4) a mixture of inactivated ascorbic acid oxidase and ascorbic acid,
5) a mixture of inactivated ascorbic acid oxidase and dehydroascorbic acid,
6) a mixture of active ascorbic acid oxidase and dehydroascorbic acid,
7) mixtures of ascorbic acid and inert protein.

Discussion

Because of the lack of an adequate theoretical basis at the present time for considering the action of ascorbic acid oxidase, any interpretation of the exchange observed between the enzyme copper and radioactive cupric ions during the enzymatic catalysis must be speculative in nature. It is not possible to decide, from the present data, whether the exchange is due to enzyme inactivation or enzyme action, or both. However, one point is clear. The "resting" (non-functioning) enzyme does not undergo exchange. This is the strongest argument yet advanced in opposition to the view that ascorbic acid oxidase may be a dissociable complex involving ionic copper and non-specific protein or other colloidal matter. The electrophoretic and ultracentrifuge data are in good agreement with the view that ascorbic acid oxidase is a specific copper protein having a molecular weight of about 150,000 and containing six copper atoms per molecule.

It is clear that before a complete understanding of the mechanism of action of such copper oxidases can be enjoyed, more information must be obtained concerning the reaction inactivation process. In the case of ascorbic acid oxidase it is now known that the inactivation is not due to an irreversible loss of copper from the enzyme. It may be that the enzyme becomes inactive as the result of some structural rearrangements within the protein during the catalytic process. During such rearrangements, the enzyme copper may become more accessible to contact with and displacement by radioactive copper. This offers a plausible explanation of the observed exchange in terms of a dependence on the reaction inactivation process.

An equally attractive explanation of the observed exchange can be developed in terms of a dependence on the enzyme activity, if it be assumed that

1) the copper of ascorbic acid oxidase is initially in the divalent state;

2) during enzymatic catalysis, the copper of the enzyme shuttles reversibly between the divalent and monovalent states;

3) the exchange occurs only when the copper of the enzyme appears in the monovalent state.

The first two assumptions are not new. Previous workers have explained the role of the copper in the copper oxidases in such terms (15, 36), and the results of carbon monoxide inhibition studies are in agreement (7, 21). The previously described color transformations of the purified enzyme, i. e., from a blue-green to yellow-brown in the presence of ascorbic acid, are also in agreement.

The third assumption—limiting the exchange to the monovalent state of copper—is based on a consideration of the structure and bond strengths of both di- and mono-covalent complexes of copper.

Wells (42) and Pauling (26) have pointed out that the usual type of covalent complex formed by divalent copper is one with a coordination number of four, having a square coplanar configuration for the directed valences of the copper atom. On the other hand, mono-covalent copper with a coordination number of four exists in a tetrahedral configuration. The point of interest here is that the square coplanar bonds are considered to be much stronger than the tetrahedral bonds (26). This implies that at the site of the attachment of the copper to the protein, the copper bond changes from a strong coplanar configuration to a weaker tetrahedral configuration each time the copper is reduced from the cupric to cuprous state. With this change to a weaker type of linkage, the possibility of an exchange with radioactive copper is enhanced.

Some experimental evidence in support of this hypothesis is available. Johnson and Hall (10) have recently reported that covalent nickel compounds of the square planar type do not exchange their nickel with radioactive nickelous ions in solution, whereas the tetrahedral nickel complexes rapidly undergo exchange. They attribute the difference in behavior toward the exchange reaction directly to the difference in bond strengths of the two types of complexes.

An important point that must be kept in mind is the fact that no exchange was observed between the copper of the enzyme and the radioactive copper ions in the presence of ascorbic acid except when oxygen was present. In terms of the thought expressed above, this fact implies that the enzyme copper undergoes a reversible oxidation and reduction cycle only when the electron donor (ascorbic acid) and the electron acceptor (oxygen) are both present. A suggestive picture, in harmony with such a stipulation, is that the formation of a ternary complex involving oxygen, enzyme, and ascorbic acid may be a prior requirement for ascorbic acid oxidase action.

The observations in regard to the effect of oxygen and of ascorbic acid on the color of highly purified ascorbic acid oxidase can be interpreted in terms of such a ternary complex. It would appear that the blue-green color of ascorbic acid oxidase may be due to a dissociable binary complex involving the enzyme and oxygen. A reaction between this complex and ascorbic acid would involve, at least in transition, a ternary complex. Such a complex would greatly facilitate the flow of electrons from the ascorbic acid to the oxygen through the copper of the enzyme. The oxidation state of the copper and the color of the complex at any moment would presumably depend on the relative amounts of ascorbic acid and oxygen available to the enzyme.

Unless it be assumed that ascorbic acid oxidase preparations from different sources may differ very markedly in their properties and molecular composition, one cannot accept Tadokoro's claim to having isolated ascorbic acid oxidase in the form of a crystalline nucleoprotein (37, 38). The homogeneous blue-green oxidase preparation described in this communication contained no phosphorus, had a nitrogen content of 16.8 per cent and showed no evidence of an adsorption band at 260 mμ.

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DISCUSSION

Dr. CHANCE: Is it possible to convert the specific data which you have given on the ascorbic acid oxidase activity into molecules of ascorbic acid oxidized per copper atom per second? Also, how many molecules of ascorbic acid can be oxidized by the enzyme before inactivation occurs? This number might be very important for comparative purposes with other enzymes that are studied as precisely. I also would like to ask your ideas concerning the nature of the copper in the blue-green form and the yellow form of the enzyme, and the state in which the copper exists in the enzyme when it forms a complex with ascorbic acid.

Dr. C. R. DAWSON: A calculation of the number of ascorbic acid molecules oxidized per enzyme molecule depends upon several factors, since the inactivation is dependent on enzyme purity, pH, temperature, etc. We have the data available, but I do not have the calculations with me.* As to the valence state that you mentioned, my concept is that the enzyme in air exists as a cupric-protein-oxygen complex which is blue-green. In the absence of oxygen, a cuprous-protein complex may explain the yellow color. In the presence of a relatively large amount of ascorbic acid the cuprous form of the enzyme also probably exists. During the repeated shuttle between cupric and cuprous, something happens to the enzyme. What it is, nobody knows. It would be of interest, however, to take the inactivated enzyme and compare it electrophoretically to the active enzyme, but the problem here is to get the quantity of the enzyme necessary to carry out this experiment.

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* Based on the initial reaction velocity, a specific activity of 2000 units/mg. corresponds to an oxygen uptake of 20,000 mm.^{3} per min. per mg. of enzyme. This corresponds to the oxidation of 1.64×10^{-3} moles of ascorbic acid per min. per mg. of enzyme. With an enzyme molecular weight of 150,000 and 6 copper atoms per molecule, the moles of ascorbic acid oxidized per mole of enzyme is 246,000 per min. The corresponding figure per copper atom is 41,000 moles of ascorbic acid per min. An inactivation total was not determined on the enzyme described in this communication. However, an enzyme of somewhat lower purity (Sp. activity = 1680 units/mg.) gave an inactivation total of 109 mm.^{3} oxygen uptake for 1.49×10^{-3} mg. of enzyme in a reaction system containing 2.5 mg. of ascorbic acid at 25° C and pH 5.6 [Powers and Dawson, *J. gen. Physiol.*, 27: 181 (1944)]. This corresponds to the oxidation of 938,000 moles of ascorbic acid per mole of enzyme before complete inactivation, or 156,333 moles of ascorbic acid per copper atom.

Dr. CHANCE: The enzyme as it stands consists of a cupric-oxygen ascorbic acid complex; is that correct?

Dr. C. R. DAWSON: That's what we believe, yes. There is no exchange with radioactive copper in this state.

Dr. NELSON: Does the radioactive copper have to be in a certain valence state for the exchange to occur?

Dr. C. R. DAWSON: It apparently makes no difference. The data of other investigators show that cuprous radioactive copper and cupric radioactive copper are interchangeable in exchange experiments.

Dr. GRANICK: Is the inactivation of the enzyme of the general character that you would expect if it were inactivation due to hydrogen peroxide? And would it not be possible that small amounts of hydrogen peroxide, or more likely some intermediate, might be formed during the oxidation, escaping from the enzyme site and that this could be the inactivating agent?

Dr. C. R. DAWSON: That's a good point. It depends upon how much hydrogen peroxide it would take to inactivate the enzyme. The experiment has been tried by adding known amounts of hydrogen peroxide to the system. The results are in favor of the view that the inactivation is not due to hydrogen peroxide, *per se*. Several years ago we conducted some experiments in which crystalline catalase was added to the enzyme-ascorbic acid system. A marked protection against inactivation was observed. The initial rate of oxygen uptake was not changed by the presence of catalase, and neither was the total oxygen uptake for complete oxidation of the ascorbic acid. If hydrogen peroxide were being decomposed by the catalase, one would expect a return of oxygen to the system, just as is observed when catalase is added to the cupric ion—ascorbic acid system which does generate hydrogen peroxide. We concluded that hydrogen peroxide, *per se*, was not the inactivator, but some transitory "redox" form of oxygen, rapidly decomposable by catalase without upsetting the oxygen balance, might be responsible.

Dr. COMAR: What is the significance of the 30% figure in your exchange data? Is that concerned entirely with the amount of copper in the radioactivity added compared with the amount of copper originally present, or is there a physiological significance?

Dr. C. R. DAWSON: I don't know what the significance of the 30% exchange is. Other experiments must be done before we can make even a reasonable guess. It might conceivably be that 30% corresponds in some way with the inactivation that occurs.

Dr. COMAR: I was thinking of the amount of copper added. In other words, if you add more radioactive copper do you get a greater exchange?

Dr. C. R. DAWSON: That experiment has not been done, but it should be. The kinetic data on exchange may well vary with the amount of copper present.

Dr. COMAR: Were the exchange studies made under conditions where the enzyme would be 100% inactivated?

Dr. C. R. DAWSON: In several cases the inactivation was practically complete.

Dr. ARNON: I was wondering if you thought of trying this peroxide idea with a catalase and alcohol system?

Dr. C. R. DAWSON: We tried that. It didn't work.

Dr. CHANCE: I would like to point out that in postulating a copper-ascorbic acid-oxygen-complex the question of formation of peroxide is no longer relevant. There may be a peroxide formed in the complex which, by a second catalytic reaction, oxidizes ascorbic acid; under these conditions it would be rather difficult to predict the nature of the catalase effects. A small amount of peroxide could form in the complex without being affected by catalase. When you utilize the concept of a ternary complex, the arguments about whether hydrogen peroxide is formed or not, based upon catalase experiments, are no longer valid.

Dr. C. R. DAWSON: That's right. Another significant point about the catalase protection is that other porphyrins will do the same thing. We have used methemoglobin, hemoglobin, and peroxidase. We feel certain that there is no hydrogen peroxide per se that develops in the reaction. There may be a very rapid transitory formation of a peroxide in the complex, however; but this does not affect the speculation above. I would like to point out, also, that our hydrogen peroxide—catalase experiments were published six years ago. The ternary complex viewpoint which I have just expressed is new and has not yet been published. I think Dr. Chance is perfectly correct that if we are to use this as a basis of speculation then all the arguments which were previously advanced concerning lack of the formation of hydrogen peroxide are relevant only in so far as they are concerned with hydrogen peroxide as a final reaction product. The effect of catalase is an experimental fact. There is no free hydrogen peroxide appearing in the reaction mixture as the result of the enzymatic oxidation of ascorbic acid. Hydrogen peroxide is formed, however, when one oxidizes ascorbic acid by copper.

Dr. GRANICK: If one adds hydrogen peroxide and ascorbic acid to the enzyme under anaerobic conditions, will there be a direct oxidation of ascorbic acid?

Dr. C. R. DAWSON: No.

Dr. KAPLAN: Is the inactive enzyme more susceptible to the removal of copper by dialysis?

Dr. C. R. DAWSON: There is no loss of copper from the enzyme protein during the reaction inactivation process. This was demonstrated by dialysis experiments and by experiments involving the ion-exchange resin.

Dr. COLEMAN: Do you have any data on the spectrophotometric characteristics of the compounds, particularly for comparison with other copper complexes?

Dr. C. R. DAWSON: No, we do not.

Dr. REDFIELD: As I understand it, after Kubowitz removed copper from his enzyme with cyanide, he could restore the complete enzyme either with cupric or with cuprous salts. With your enzyme, if you replaced copper with cuprous copper is your enzyme active?

Dr. C. R. DAWSON: Dr. Joselow, who did this work, repeated Meiklejohn and Stewart's experiment using ascorbic acid oxidase and copper sulfate. It is an experiment that is difficult to do, as you can imagine. Unfortunately, he did not attempt to restore the enzyme activity with cuprous salts. He was not interested in this particular point at that ime.

Dr. COMAR: One has to be a little careful in interpreting the exchange data inasmuch as in order to get an exchange all that is necessary is to have an instantaneous release of the copper from the complex and its return to the complex; and if that happens, regardless of the valence state, you will get an exchange. You don't have to postulate, therefore, a complex in which the copper is less tightly bound. The exchange could occur if you had instantaneous release of the copper in any kind of complex.

Dr. C. R. DAWSON: Surely. The thing that we have to explain is the fact that the enzyme by itself does not exchange; the enzyme plus substrate under anaerobic conditions does not exchange, and the enzyme plus products of the reaction does not exchange. The only time we obtain an exchange with the isotopic copper is during catalytic activity.

Dr. Comar: One might postulate that the conditions which you have in the presence of the substrate would give you instantaneous release and therefore would allow for an exchange with the isotopic copper in the medium.

Dr. C. R. Dawson: I think that these viewpoints may be essentially equivalent and that you are merely postulating a mechanism for the exchange. I see no objections to this viewpoint but believe that additional experiments are necessary before it can be established.

Dr. Comar: I don't believe that the viewpoints are quite the same. As I understand it, you are saying that in your cuprous complex the copper is less tightly bound and therefore will exchange.

Dr. C. R. Dawson: I am not saying that that is the case. I am only suggesting it as an explanation on the basis of evidence which has been obtained from studies using isotopes other than copper. For example, radioactive nickel has been shown to exchange with nickel in a complex more readily in one valence state than the other. So far as we can tell, these states are comparable to those we have encountered in the present experiments.

THE NATURE OF THE COPPER ENZYMES INVOLVED IN TYROSINE OXIDATION

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For various reasons copper enzymes have attracted widespread attention for over half a century. The resulting data have been reviewed most recently by Nelson and Dawson (71) and Dawson and Mallette (45), the latter considering the tyrosinase enzymes from the point of view of copper proteins. In general, the enzymes from various sources that will be considered here are characterized by their ability to catalyze the aerobic oxidation of a wide variety of both phenol and catechol derivatives. In the present review it has been possible only to select arbitrarily from among the numerous references dealing with enzymes of the tyrosinase type.

The early work of Bourquelot and G. Bertrand (26) on mushrooms led to the observation that injury results in alterations of pigmentation of certain species through red to dark brown or black. Such behavior contrasts with a blue stage produced in some mushrooms by the oxidase laccase and indicates the presence of a different enzyme system. Bourquelot and Bertrand were able to separate a substance that is aerobically oxidized by the mushroom juice to a soluble red and finally to a black material resembling the pigment melanin. This substrate was shown by Bertrand (11) to be tyrosine, and subsequent studies have led to extensive work on the mechanism of the reaction and the nature of the catalytic agent, called tyrosinase by Bertrand. Since his time a variety of names has been given to the enzyme, a confusion that arose from differences between enzyme preparations obtained from different sources and in different ways. Included among these names are phenolase, polyphenol oxidase, dopa oxidase, catecholase, etc., based upon a particular substrate. The extent to which these terms are interchangeable has not yet been fully determined. The difficulties involved in the comparison of preparations are, at least in part, a reflection of difficulties in the purification of the enzyme.

Copper in the Enzyme

Difficulties of preparation were in a large measure responsible for failure to recognize early in the work that the enzyme tyrosinase contains essential copper. This was shown to be true by Kubowitz (56) in 1938, by the demonstration of a relation between the copper content and the catalytic activity of the preparation toward catechol. These classical studies involved the use of a variety of copper inhibitors and, in addition, removal of the copper by dialysis in the presence of cyanide. The activity loss resulting from the removal of copper could be almost completely reversed by the addition of excess cupric ion. On the contrary, activity was not restored by other divalent metals, such as iron, cobalt, nickel, manganese, or zinc. Allen and Bodine (1) also found by copper removal experiments that protyrosinase from grasshopper eggs requires copper for conversion into active tyrosinase, and that other divalent heavy metals cannot be substituted for copper. Tenenbaum and Jensen (92), in studying the effect of copper inhibitors on mushroom tyrosinase, reported reactivation of the enzyme by heavy metal ions. However, these workers did not actually remove the copper, a requirement in replacement studies, since Webb and van Heyingen (97) and D. Bertrand (10) found in studies of tyrosinase inhibitors that added heavy metal combines with the inhibitor and restores the enzyme activity. In these and earlier inhibitory experiments a wide variety of inhibitors of tyrosinase have been observed, including cyanide and carbon monoxide (56), 2, 3-dimercaptopropanol (10, 97) and a great number of other compounds containing sulfur (30, 37, 40, 75, 81).

Reports of the copper content of the tyrosine oxidases vary, depending apparently on three factors: (a) enzyme source, (b) degree of purification, and (c) method of purification. Some of the data reported on various preparations of high activity are summarized in Table 1. The methods of assaying activity vary to such an extent that quantitative comparisons cannot be made. The possible effect of the methods of preparation on the absolute copper content will be considered later.

Kubowitz (56) concluded from his inhibition studies that the enzyme, as isolated, contained cupric copper, and he postulated the catalytic oxidation of catechol as:

$$4\ Cu^{++}(\text{enzyme}) + 2\ \text{catechol} \longrightarrow 4\ Cu^{+}(\text{enzyme}) + 2\ \text{o-quinone} + 4\ H^{+}$$

$$4\ Cu^{+}(\text{enzyme}) + 4\ H^{+} + O_2 \longrightarrow 4\ Cu^{++}(\text{enzyme}) + 2\ H_2O$$

It might be well to emphasize that so far this hypothesis remains unproved, largely because of preparational difficulties.

TABLE 1

THE COPPER CONTENT OF SOME EXTENSIVELY PURIFIED TYROSINASE-TYPE ENZYME PREPARATIONS

Copper content	Source	Relative catecholase activity*	Reference
0.2	white potato	....	Kubowitz (56)
0.3	cultivated mushroom	quite high	Keilin and Mann (52)
0.1	"	high	Parkinson and Nelson (74)
.23	wild mushroom	low	Dalton and Nelson (32)
.08	tea leaves	....	Sreerangachar (90)
.028	cultivated mushroom	low	Mallette et al. (62)
.10	"	high	Mallette and Dawson (61)
.21	"	quite high	Mallette et al. (62)

* Indicating a high activity of the enzyme toward catechol as a substrate as compared with the activity toward the substrate p-cresol. These two activities have often been named catecholase and cresolase.

Occasional reports have been made of oxidase activity on the part of non-specific complexes of protein and copper. For example, Bhagvat and Richter (14) were able to identify such action of certain bloods as due to the hemocyanins of these bloods. In addition, artificial complexes of copper with various proteins show oxidative activity toward catechols (91) and adrenaline (27). It seems that such systems attack catechols but not monophenols, and that the activity is very low when compared to the true tyrosinases on the basis of the protein or copper present.

Occurrence of Tyrosinase-type Enzymes

Such enzymes occur widely in nature, often in conjunction with laccase, peroxidase, and catalase, which may influence or modify their activity. The enzymes are principally known in plants and invertebrates, with a much less general distribution among the higher animals. The plants containing the tyrosine oxidases include many fruits and vegetables which darken upon injury, e. g., tea, mushrooms, beans, wheat, artichokes, fungi of several genera, spinach, apple leaves, and certain bacteria. Lower animals in which these enzymes have been reported include cuttlefish, certain crustacea, and a number of insects and some larvae. Various enzymes which may fit into this category have been observed in melanotic tumors, certain normally pigmented skin areas of mammals, feather germs, and guinea pig livers. In view of the great number of reports on the occurrence of the tyrosinases, it does not appear advisable to catalog them in detail. Suffice it to say that they make up one of the most extensively distributed groups of enzymes in nature.

Enzyme Preparation

In the usual source the enzyme is very dilute (perhaps as a consequence of a comparatively high activity), and the problem of crystallization and complete purification has never yet been successfully concluded. The usual methods are those commonly employed in enzyme purification and include cell rupture, as by freezing, removal of the natural substrates, often by acetone extraction, and subsequently the customary fractionations with salts, adsorption, etc. Among the more extensive purification studies have been those of Kubowitz (55, 56) on white potato; of Keilin and Mann (52), Ludwig and Nelson (59), Parkinson and Nelson (74), and Mallette et al. (62) on the common mushroom, *Psalliota campestris*; of Dalton and Nelson (32) on a wild mushroom, *Lactarius piperatus*; of Sreerangachar (90) on tea leaves; of Hogeboom and Adams (51) on mouse melanoma tumor; and of Eiger and Dawson (43) on sweet potato.

The preparation by Dalton and Nelson (32) yielded a small proportion of its total weight in crystalline form, and these crystals

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contained 0.25 per cent copper; but they were only slightly soluble, and the recovery of the activity was low. These authors demonstrated a correlation between the crystals and enzyme activity, but the loss of solubility and the recoverable activity indicated some interference by denaturation, so that the problem is not yet finally solved.

Mallette and Dawson (61), testing highly purified mushroom tyrosinase preparations, found them to be still slightly heterogeneous in the only study so far reported on purity, per se. The preparations investigated (62) were very high in activity, and if compared with others on a Q_{O_2} basis for the oxidation of catechol, exceeded that of Keilin and Mann (52) by a factor of three. The latter exceeded the potato preparation of Kubowitz (56) by twenty times, which in turn is ten times as active as the tea oxidase prepared by Sreerangachar (90). It should be made clear, however, that activity assay methods greatly influence Q_{O_2} data, and such comparisons, although frequently made in the past, mean little.

The unusual difficulty in preparation seems in part to be due to a partial fragmentation of the enzyme-protein molecule that appears to occur quite readily during the early stages of purification. This somewhat unique concept will be developed more fully later under a consideration of the nature of the enzyme. Suffice it to say here that if such fragmentation does occur, the effect will be a further complication of the purification process already complicated by the presence of a maximum of only 5 to 10 mg. of enzyme per pound of mushrooms, which is a " rich " source. In spite of this difficulty, however, preparational investigations have led to a problem of theoretical interest extending to enzymes and proteins in general, namely, the possibility of losing portions of the protein entity when it is removed from its natural location and separated from related materials.

Substrates for Tyrosinase Enzymes

As is customary among enzymes, a degree of specificity is manifested by this group of enzymes taken from various sources in various ways. In general, the substrates of enzymes of the tyrosinase type are derivatives of phenol or of catechol. Hydroquinones and resorcinols are not catalytically oxidized by the enzymes of this group. As indicated by the name tyrosinase, the amino acid tyrosine is oxidized by the enzyme in the presence of air (Bertrand (11) and many later workers). Bertrand (12) found activity of the enzyme on a number of other phenols, such as phenol, p-cresol, p-hydroxyphenyl acetic acid, etc. Among the o-dihydroxybenzene derivatives whose oxidations are catalyzed by tyrosinase are catechol, pyrogallol, β-(3, 4-dihydroxyphenylalanine), adrenaline, protocatechuic acid, etc. (see Nelson and Dawson (71) for additional details). Of the more recent studies of substrates mention should be made of the work of Beyer (13), Randall and Hitchings (78), Enselme and Vigneau (46), Cushing (31), and Enselme and Petat (45). Since the enzymatic rate varies with the substrate, correlations of activity and structure have been made by some of the preceding authors.

The natural substrates identified with the enzyme include tyrosine (11), dopa (82), tea tannins (21, 22), and chlorogenic acid (84). Bergstrom, Theorell, and Westman (9) have correlated potato enzyme activity with destruction of estradiol, while Sizer and Prokesch (89) have observed oxidation of the active principles of poison ivy by tyrosinase. Furthermore, they claim prevention of the skin reactions if tyrosinase is applied in the early stages. Since the allergic response seems to occur only after the active agent has passed through the skin, it is difficult to see how the protein can have any effect except on the outer skin surface.

Sizer (86-88) has reported that tyrosinase attacks yeast invertase, trypsin, chymotrypsin, and pepsin, presumably through the tyrosine of the protein molecules, with at least partial loss of the activity of the protein substrates. Edman (41, 42) has contended that the results observed were due to free tyrosine in the substrate proteins and that loss of activity by these proteins was not due to the tyrosinase.

The features involved in the two different types of oxidation reactions, namely, the oxidation of phenol and of catechol, will be considered separately although they are not completely independent. Other reactions observed subsequent to the phenol-catechol are of sufficient interest to warrant consideration in another section.

Phenol Oxidations

All available evidence supports the concept that the monophenol is oxidatively converted by enzyme into the catechol, as suggested by Raper (79). Proof has been somewhat difficult, however, because of the possibility of oxidation of monophenol by subsequent products rather than by the enzyme and oxygen. Several arguments have been brought to bear on this point, namely: (1) Bordner and Nelson (25) excluded H_2O_2 as the possible oxidizing agent; (2) both they and also Pugh (76) enzymatically oxidized catechol to o-quinone, which in turn was found to have no effect on p-cresol when added to it; (3) addition of benzenesulfinic acid removes o-quinone quantitatively as fast as the quinone forms, thus breaking the reaction sequence at this stage, but without preventing the enzymatic oxidation of monophenol (25, 77); (4) phenol dehydrogenase was excluded by Bordner and Nelson (75), using quinone, phenol, and enzyme under nitrogen with no phenol oxidation, with a result identical to that from various controls; (5) Kendal (53) and Behm and Nelson (7) have studied monophenol oxidation with ascorbic acid present in the system and obtained normal rates, although the reduction of o-quinone to catechol by ascorbic acid appears to be essentially instantaneous (68, 69); such a system would seem to contain no potential oxidizing agents; (6) finally, the peculiar variability of the ratio of the two activities logically excludes oxidation by reaction products which would be present in fixed amount with a given quantity of catecholase activity.

A peculiarity of the oxidation of a number of phenols is the autocatalytic nature of the initial phase of the reaction. The present view of the origin of the induction period has been developed by Nelson and Dawson (71). Apparently, oxidation of a phenol by tyrosinase and oxygen begins only when a few molecules of a catechol are oxidized so as partially to activate the phenolase portion of the enzyme. Autoxidation of a phenol will always give traces of catechol; then, as catechol is formed enzymatically from phenol, the rate of oxidation of the latter will reach a maximum when an optimum amount of the catechol undergoes simultaneous oxidation. Hence, any factor influencing the tyrosinase oxidation of catechol also affects the phenol reaction. Such factors include (1) oxidizing agents which prolong the phenol induction period very greatly by oxidizing catechol before the enzyme can do so, (2) reducing agents which have the opposite effect and keep a high concentration of catechol for enzymatic oxidation, and (3) added catechol, which will get the " priming " catechol oxidation under way at once. In addition, the lag period is lengthened and the general rate reduced by those agents which either form complexes with catechols, like boric acid, or else like the pH, which controls the water reaction of o-quinone, or materials which remove o-quinone as does benzenesulfinic acid, affect subsequent catechol formation from o-quinones. These interpretations come from observations by Bordner and Nelson (25), Pugh and Raper (77), Behm and Nelson (7), and Dawson and Nelson (36).

Implicit in the foregoing concept is the idea that if simultaneous catechol oxidation is required for the oxidation of a phenol, then a complex containing catechol, phenol, and enzyme probably exists. If so, then the natures of the particular catechol and phenol involved may influence both the induction period and the general phenol oxidation rate whenever they affect the composition of the enzyme-phenol-catechol complex. Likewise some effect on catechol oxidation would be expected, and such interferences have been observed (6, 71).

Kendal (53) has studied the problem from the point of view of the oxidation of phenols and has shown that competitive interference does exist when two substrates are present. The disappearance of the induction period when a catechol or ascorbic acid is added, Kendal attributed to some sort of an activation of the monophenolase and not merely to an accumulation of o-diphenol. He did not feel that oxidation of o-diphenol is a necessary process for this activation and believed that lag periods in oxygen uptake are due to the slow accumulation of significant amounts of catechol, which are then oxidized independently. This view disagrees, however, with the observation of Behm and Nelson (7) that there is a lag period when the reaction is followed by observing the rate of disappearance of phenol, a rate which agrees very closely with that of O_2 uptake.

Kendal also showed, by following catechol concentration, that phenol is oxidized at a maximum rate at the initiation of the reaction, and that the rate slowly but continuously diminishes, apparently by inactivation of the enzyme. It might be well to point out here, however, that the oxidation of the catechol occurs simultaneously with that of the phenol in his system and that this process is known to cause inactivation when proceeding by itself. It is difficult, therefore, to judge whether the inactivation reported by Kendal is due to phenol oxidation, or only to catechol oxidation. The data shown are exceedingly like those for the oxidation of catechol alone, as demonstrated in his work and that of Ludwig and Nelson (59) and Miller et al. (69). Kendall (53) believed that the initial induction period in the oxidation of phenols (as followed by oxygen uptake) is due to inhibition of the oxidation of the catechol by the phenol.

Onslow and Robinson (73) in early work felt that monophenols were oxidized by o-quinones, a view later taken up by Califano and Kertesz (28) when they observed elimination of the induction period through the addition of catechol. Kertesz (54) recently still adhered to his view and supported it by an elaborate kinetic study. How he accounted for changes in the activity ratio and complete removal of o-quinone by rapid reduction or precipitation is not clear.

Catechol Oxidations

The first step in the oxidation of catechols is in general not so complex as that of phenols, and involves removal of hydrogen atoms from the two adjacent hydroxyl groups with formation of water and the corresponding o-quinone. The possibility of the formation of H_2O_2 has been excluded by Dawson and Nelson (36) and Dawson and Ludwig (34), and tyrosinase is therefore a "true oxidase."

Apparently as a consequence of this reaction, however, many preparations show a loss of activity while oxidizing catechol. This inactivation results in a steady decrease in the rate and is pronounced when the preparation used is relatively high in catecholase activity. Such loss of activity has not so far been reversed once it has occurred, but is not observed with all catechols nor with cresolase preparations high in activity. Subsequent reaction products have been ruled out as a cause of this inactivation by using ascorbic acid, which instantaneously reduces o-quinones back to catechols and keeps the catechol concentration constant while the ascorbic acid itself continuously disappears. A scheme for following the disappearance of ascorbic acid has been developed into a catecholase assay method by Miller et al. (69), who mathematically obtained the rate at zero time, before an enzyme inactivation had occurred.

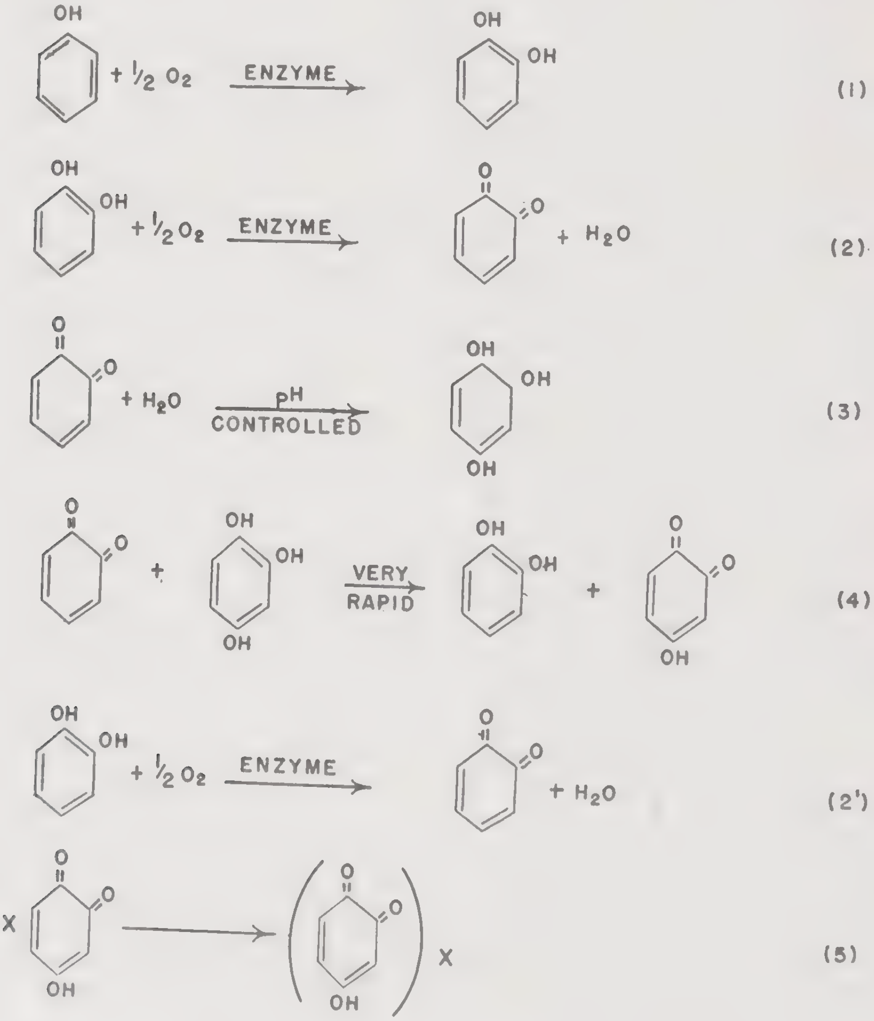
In general, the various substrate catechols are oxidized at somewhat different optimum rates, and the optimum substrate concentrations vary with catechol structure. Furthermore, the rate of inactivation of the enzyme catalyzing the reaction varies with the catechol. Roth (83) found no simple correlation between these reaction properties and the catechol structure. His study was based on systems containing ascorbic acid and has apparently excluded both reaction products and unstable intermediates involving oxygen and water as possible causes for the reaction inactivation of tyrosinase.

Recently Asimov and Dawson (4) have concluded that the inactivation is not due to surface phenomena nor to the momentary presence of o-benzoquinone or related compounds. They have shown the reaction to be of a 3/2 order and feel that a stepwise process results in a loss of activity by stages and finally yields inactive enzyme. These kinetic investigations led to modifications in the methods of assaying catecholase activity and were extended until enzyme inactivation was over 95 per cent complete, so as to permit a determination of the actual order of the reaction.

The influence of phenol on the oxidation of catechol has been examined (6) and the nature of the substrate system found to control the rate of oxidation of the catechol. For example, the degree of inhibition of the oxidation of catechol itself by a given amount of phenol is much less than the inhibition of the oxidation of 4, 5-dimethylcatechol by 3, 4-dimethylphenol. In the cases studied, the oxidation rate of the catechol is reduced when the phenol is added. However, when the concentration of the catechol, with phenol present, is increased beyond the concentration optimum for catechol alone, the rate increases and apparently approaches the rate of oxidation obtained when using an optimum amount of catechol without phenol. The inference seems to be that there exists a competitive inhibition for the " active sites " on the enzyme, with some sort of equilibrium and mass law conditions applying to the system.

The Products of Tyrosinase Oxidations

The present view of the oxidation of dilute solutions of phenol by mushroom tyrosinase and oxygen is summarized in the reactions:

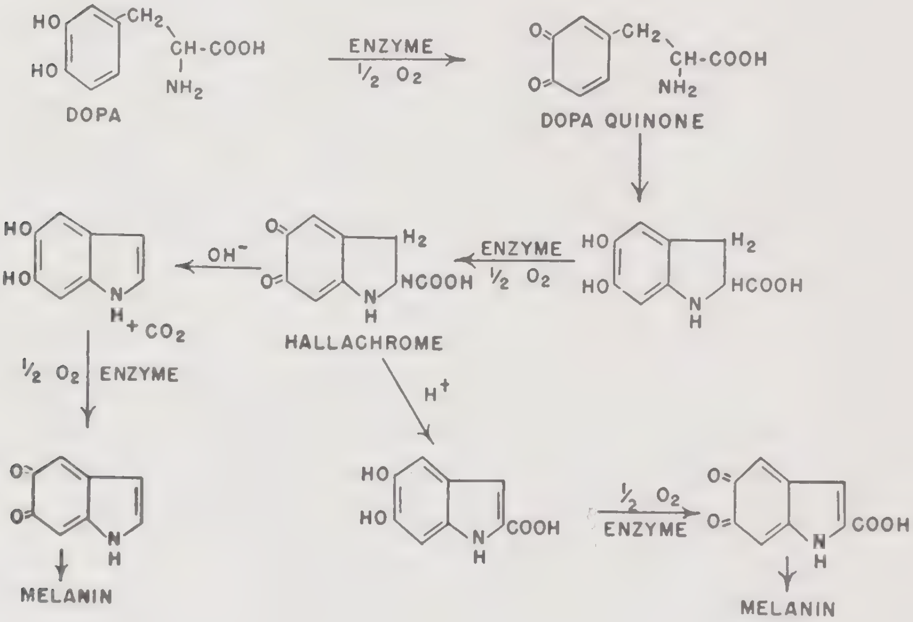

OH
+ ½ O2
ENZYME
OH
OH
(1)
OH
OH
+ ½ O2
ENZYME
O
O
+ H2O
(2)
O
O
+ H2O
pH CONTROLLED
OH
OH
OH
(3)
O
O
+
OH
OH
OH
VERY RAPID
OH
OH
+
O
O
OH
(4)
OH
OH
+ ½ O2
ENZYME
O
O
+ H2O
(2')
X
O
O
OH
O
O
OH
X
(5)

Reactions 1 and 2 have already been discussed. Dawson and Nelson (36) found that o-benzoquinone reacts with water at a rate controlled by pH, and Wagreich and Nelson (94, 95) indicated that one of the compounds formed in the seqeuence was a hydroxy-o-benzoquinone, probably the 4-hydroxy derivative. Subsequently they investigated the system further and concluded that catechol was reformed by means of reactions 3 and 4 and then reoxidized (2′), resulting in the consumption of a third atom of oxygen. The o-benzoquinone formed by the enzyme is thus continuously converted into 4-hydroxy-o-benzoquinone, which polymerizes rapidly to pigments. Other evidence supporting this scheme was cited by Nelson and Dawson (71).

Recently, Mason (66) has examined the enzymatic oxidation of catechol spectrophotometrically and has verified the formation of o-benzoquinone as the initial reaction product. He also obtained spectrophotometric evidence for the formation of catechol in aqueous solutions of pure o-benzoquinone and has actually isolated catechol. However, because he was unable to find spectrophotometric evidence for the existence of hydroxyhydroquinone or hydroxy-p-quinone during the course of the enzymatic oxidation of catechol and because under the conditions of his experiments the catechol-enzyme system was observed to take up more than two atoms of oxygen per molecule of catechol oxidized, Mason criticized the mechanism given above and suggested "that o-benzoquinone polymerized directly to phenolic polyphenols susceptible to further oxidation." In making this suggestion, Mason has completely overlooked the fact that o-benzoquinone, as measured spectrophotometrically or iodometrically, disappears more slowly in aqueous solutions containing the enzyme and oxygen than in the absence of the enzyme.

The results of the recent studies by Tarpley and Dawson at Columbia University (personal communication) reveal that the concentrations of hydroxyhydroquinone and hydroxyquinone involved in the enzymatic oxidation of catechol are too low to be detectable by the spectrophotometric technique employed by Mason. The arguments used by Mason, therefore, in criticizing the above mechanism are not valid. Furthermore, apparently through some misunderstanding, Mason has based much of his argument on the unlikelihood of hydroxy-p-quinone being an intermediate, whereas it is the hydroxy-o-quinone that was proposed as an intermediate by Wagreich (93) and by Nelson and Dawson (71).

The oxidation of tyrosine and dopa by tyrosinase has been very widely investigated because of the end product, melanin, an important pigment. The early work by Raper and his coworkers (80) established the general nature of the reaction sequence, which is presented below as modified and summarized by Mason (65).



The details in the development of this reaction system will be left to the references noted, except to point out that Mason (64, 65) and Mason and Wright (67) have completely verified the presence of the quinone stages and added to the early work the "branch" in the system occurring when the hallachrome rearranges one way in basic solution and another in acid.

THE CHARACTERISTICS OF TYROSINASE ENZYMES

Protyrosinase

We turn now from catalytic properties to the general characteristics of tyrosinase enzymes. Bodine and his coworkers (15) have made an extended study of a material which was isolated from grasshopper eggs and which when activated yields active tyrosinase capable of oxidizing phenol and catechol derivatives. The inactive may be converted to the active enzyme by a variety of treatments including the action of heavy metals (18) or detergents (2, 17), or shaking (2). Development of the tyrosinase precursor has been correlated with the egg stage, and concepts of its location and formation have been developed (16, 19). This work is an important contribution to our knowledge of enzyme precursors and indicates a parallel with the well known precursors of the hydrolytic enzymes.

Dual Nature of Mushroom Tyrosinase

A concept important to enzyme chemistry, and one which has been much debated, has developed from the work on the tyrosinase enzymes, namely, the idea that an enzyme may catalyze two distinctly different reactions. This concept has recently been extended to other systems, for example, to both ester and peptide hydrolyses by chymotrypsin and related hydrolytic enzymes. In the case of tyrosinase, where the idea first arose, the serious preparative obstacle made it hard to obtain a homogeneous protein and thus to rule out the possibility of a mixture of two copper enzymes. In addition, the variations in the ratio of the two activities found in different preparations indicated the existence of two separable enzymes, and this view was widely held until various conflicting pieces of evidence gave rise to the theory of the enzyme's dual nature. The discovery of " dopa oxidases " from animal tissues which appear to have no effect on tyrosine has further confused the issue, but of these enzymes too little is known to permit careful comparison with the copper proteins obtained from potato, mushroom, etc.

Solution of the problem was undertaken by Mallette and Dawson (61) through purification and study of the resulting preparations.

Homogeneity studies, made whenever the amount of available enzyme permitted, involved an electrophoresis analysis of five preparations with pH variation on two of them. One preparation was, in addition, ultracentrifuged. Data on activity, dry weight, and copper content completed the experimental results obtained from these mushroom tyrosinase preparations, which represented a high cresolase, a high catecholase, and a somewhat intermediate type still relatively high in catecholase activity.

All four preparations high in catecholase activity showed at most only traces of separable components by electrophoresis, and the isolated extraneous components, when present, seemed to have no enzymatic activity. The one high cresolase preparation behaved in a similar way except that the fast component formed 10 to 25 per cent of the total amount of material, but again when sampled apparently had no activity. It was concluded, therefore, that the two characteristic activities could not be separated electrophoretically, especially since runs at pH values of 5, 7.5, and 9 showed little difference. However, there seemed to be some relationship between electrophoretic mobility and ratio of activity on alternate substrates (Fig. 1). This effect was interpreted as arising during the purification procedure and is a consequence of the postulated partial degradation of the original naturally occurring enzyme molecule.

One of the high catecholase preparations was ultracentrifuged and contained 10 to 20 per cent of relatively light material, but a test of this lighter material indicated that the two activities could not be separated on the basis of size of particles.

Analytical data on the five preparations are presented in Table 2 where various correlations are also shown indicating certain conditions that must be accounted for in any structural model.

It will be observed that the data are listed according to decreasing copper content and that this order corresponds to a decreasing activity ratio for the three classes of preparations studied. The relationship turn out to be linear (Fig. 2). Secondly, in the high catecholase preparations * the catecholase activity per microgram of copper is largely independent of the activity ratio. This situation was

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* High catecholase to cresolase activity ratio.

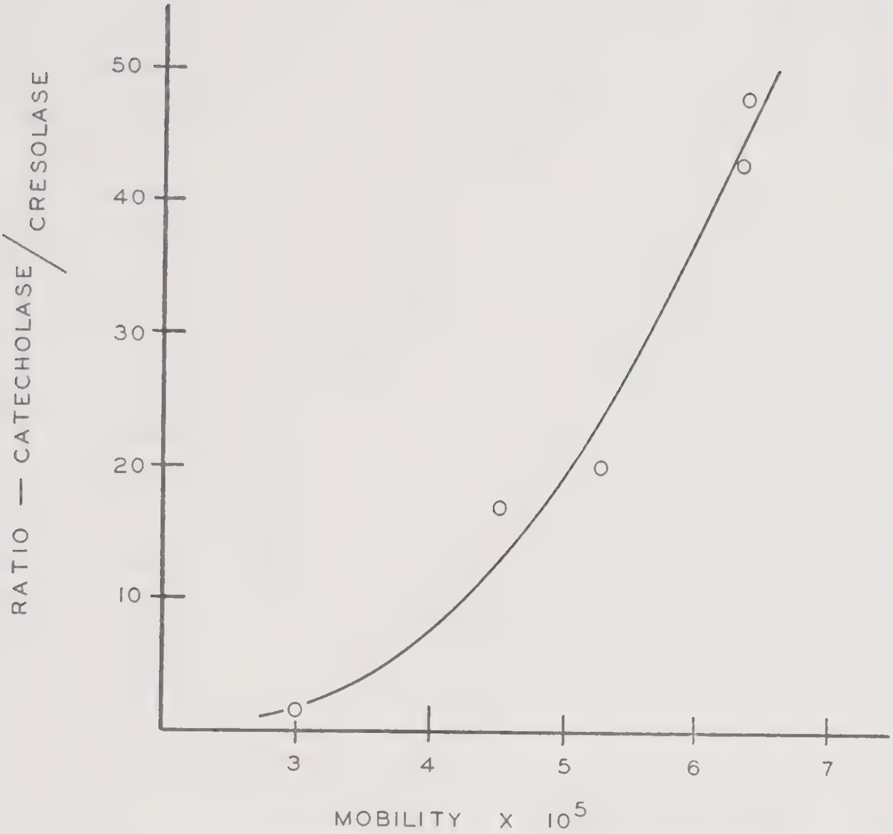

Fig. 1. The relationship between the ratio of catecholase to cresolase activity and the electrophoretic mobilities of highly purified mushroom tyrosinase preparations at pH values of 7.58 to 7.71.

TABLE 2

The Enzymatic Properties and Copper Contents of Five Highly Purified Tyrosinase Preparations from the Common Mushroom *Psalliota campestris*

Preparation	Copper %	Activity Units/ml.		Units/γCu		Units/mg. Dry Weight		Ratio of Activity
		Cat.	Cre.	Cat.	Cre.	Cat.	Cre.	Cat./Cre.
I	0.206	21,500	450	2130	48	4400	95	48
II	0.204	49,000	1140	1980	47	4050	95	43
III	0.103	11,500	570	2100	104	2150	107	20
IV	0.098	18,500	1100	2320	137	2270	135	17
V	0.028	1,880	1180	856	536	237	149	1.6

examined in detail by Ludwig and Nelson (59) who found it to be general. They further showed that the cresolase activity varies with activity ratio, as shown here. Finally, the catecholase activity per mg. of dry material roughly parallels the activity ratio, while

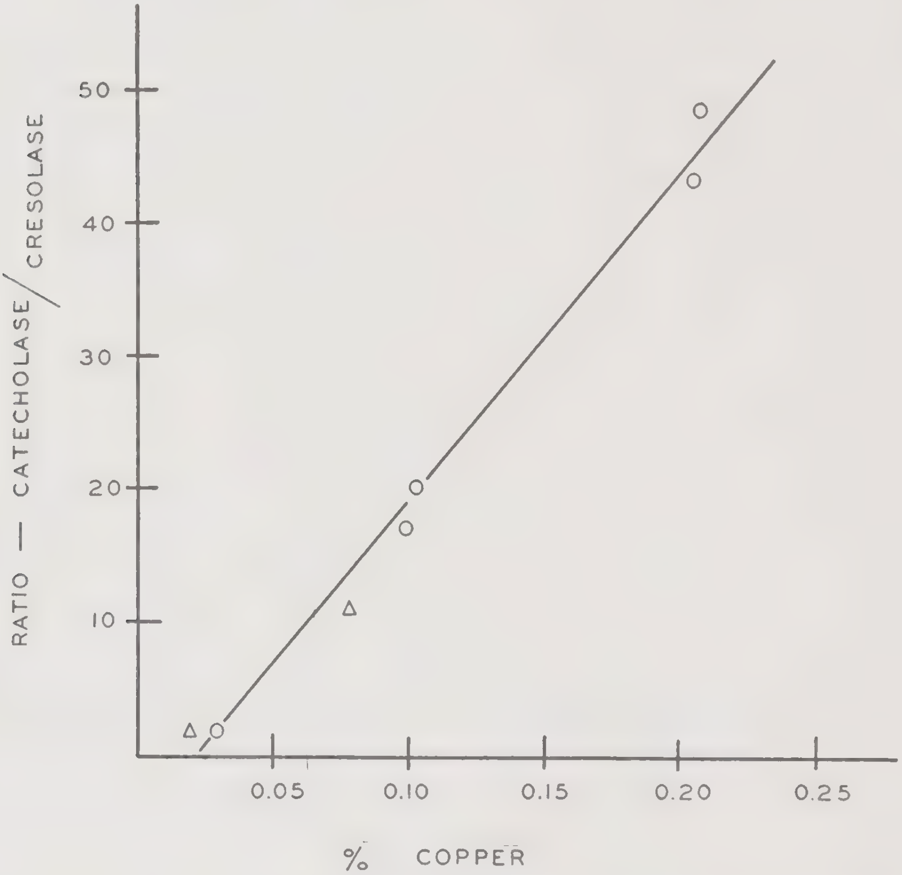

Fig. 2. The relationship between the activity ratios and copper contents of highly purified tyrosinase preparations. Triangles are data taken from Roth (83).

the cresolase activity per mg. increases slightly with decreasing ratio.

At first glance, these data when taken alone certainly suggest that a catecholase copper-containing enzyme is being freed of a cresolase copper-free enzyme, since the copper content, the specific catecholase activity, and the activity ratio all increase together. However, several points of evidence already mentioned, and some others, do not support such a view. These observations may be summarized as follows:

Electrophoresis over a range of pH gave no indication that preparations III and IV were only half as pure as I and II, or that V is 5 to 10 per cent as pure as I and II, relations which would be required by the data on the specific activity of catecholase. The copper content, like the catecholase activity per mg., indicates extensive impurity in preparations III, IV, and V as compared to I and II. Furthermore, the activities could not be separated by either electrophoresis or ultracentrifugation, nor has anyone ever succeeded in preparing a relatively pure cresolase enzyme by any means whatsoever from any of the usual sources.

It has also been suggested that the simultaneous oxidation of a catechol is necessary for the enzymatic oxidation of a monophenol (7, 25), a relation which once again would indicate a close dependence of cresolase activity on the molecule responsible for catecholase activity. Such dependence extends also to the copper known to be essential for both activities (49), in spite of data on copper content in Table 2 which suggest that there is no copper in the cresolase-active material. Finally, Kendal (53) has described the dependence of the rate of oxidation of a phenol on the presence of a catechol, and Behm and Mallette (6) have made a similar study on the effect of phenol on catechol oxidation. Both phenomena reveal a close dependence of the two reactions, and indicate only a single enzyme.

An attempt has been made (61) to fit the evidence into a hypothetical pattern, along the following lines: (1) The enzyme tyrosinase is a single protein with two characteristic activities; (2) the molecular size of the enzyme varies with the activity ratio, etc., in different preparations; (3) fragmentation and loss of portions of the enzyme molecule may occur during purification as a result of chemical or physical treatment; (4) the fragments lost contain none of the enzyme copper originally present but in some other way are important for cresolase activity.

An enzyme model having these properties makes it now possible to account for the various apparently conflicting observations already discussed. There remain some key experiments, however, to check the accuracy of this model, but unfortunately sizeable quantities of highly purified enzyme, particularly of the high cresolase type, are required, and these are difficult to obtain. Assuming the model to be valid, purification changes will produce a high catecholase type of preparation from the natural system, will cause an increase in copper content and catecholase specific activity with increasing activity ratio, alter the surface properties, make it difficult to obtain complete homogeneity, owing to a need to separate particles of similar activity but slightly different size, etc., and will yield a particle still having activities related to each other and to the same copper. It should be pointed out that changes in the activity ratio are usually greatest in the early stages of purification and that one apparently cannot convert a high catecholase into a high cresolase preparation. In general, it seems that it is cresolase activity which is subject to greater loss. For example, in the chronometric catecholase method (69), a cold dilute enzyme solution does not lose measurable catecholase activity for 30 minutes after dilution and does not deteriorate rapidly afterwards. Cresolase activity, on the other hand, is lost rapidly in the same preparation when diluted cold, and the activity has dropped to 75 per cent of the original value in 28 minutes and to 9 per cent in 95 minutes (60).

Easy loss of cresolase activity during purification has often been reported, recently by Enselme et al. (44) who chromatographically removed such activity, apparently completely, and concluded that phenolase and catecholase were distinctly separate enzymes. Eiger and Dawson (43) found little catecholase activity, as compared with phenolase activity, in untreated sweet potato slices. On treatment with sodium chloride the catecholase activity increased, and when the cells were ruptured and an extract obtained free from cellular debris, only catecholase activity remained. They were unable at the time of reporting to obtain from sweet potato an enzyme preparation capable of oxidizing phenol. On the other hand, Bodine (15), working with protyrosinase from grasshopper eggs, found that activation to tyrosinase in various ways yielded enzymes which differ in their activity ratios, although they apparently arose from a single proenzyme.

TYROSINASE AND PLANT RESPIRATION

The role played by tyrosinase as a terminal oxidase in many plants is in part responsible for the extensive study given this enzyme. Among the more recent is the investigation by Baker and Nelson (5) who found that at least 85 per cent of the oxygen uptake of respiring potato slices is catalyzed by tyrosinase. Soon afterwards, Robinson and Nelson (82) showed that the terminal oxidase system in the white potato involves tyrosinase, tyrosine, and dopa. They suggested that the dopa (dihydroxyphenylalanine) is oxidized by oxygen and tyrosinase, and that the quinone formed then withdraws hydrogen from the next segment of the respiratory chain. The tyrosine appears to function as a reservoir for supplying dopa when any of the latter is lost in any way. This idea agrees with the concept that upon injury to the cell tyrosinase action causes pigment to form and destroys its natural substrates. The overall situation may be represented as:

$$\begin{tikzcd}
\text{L-Tyrosine} \arrow[r, "1/2\ O_2", "\text{Tyrosinase}"'] & \text{Dopa} \arrow[d, shift left, "\begin{array}{l}1/2\ O_2\\ \text{Tyrosinase}\end{array}"] & \\
& \text{o-Quinone} \arrow[u, shift left, "\begin{array}{r}\text{2 H from}\\ \text{respiratory}\\ \text{system}\end{array}"] \arrow[r, "\text{interruption}", "\text{of respiration}"'] & \text{melanin}
\end{tikzcd}$$

Extending these concepts, Belval and Laeuffer (8) noted that all the living tissues of the white potato plant contain the complete system, enzyme and substrate, and that a number of related species show the same extensive distribution throughout the tissues. Schade, Bergmann, and Byer (85) rechecked the studies on natural respiration and found that in the white potato tuber synthetic substrates such as catechol can be used but damage the respiratory system at some point, although presumably not by tyrosinase inactivation.

Using sweet potato, Walter and Nelson (96) noted that tyrosine, p-cresol, phenol, and 3, 4-dimethylphenol can take part in its respiration, since slices of the sweet potato in the presence of any one of these phenols showed an increase over the endogenous respiration, and the respiratory quotients were close to unity. However, the natural substrate present in the sweet potato was not identified until Rudkin and Nelson (84) isolated it and showed it to be chlorogenic acid.

Tea leaf respiration has been attributed to a copper oxidase which apparently attacks catechol derivatives. The process involves tannins which are oxidized by the enzyme (20, 23, 57). When the normal state of the leaf is disturbed, as by injury on picking the leaf, darkening occurs by complete oxidation of the tannins and subsequent color-forming reactions. Li and Bonner (58), however, feel that the "polyphenol oxidase" of tea is not involved in the normal respiration of that plant. Bonner and Wildman (24), studying spinach leaves, suggested that dopa might be the oxygen carrier and be coupled to the other respiratory systems by acting as a hydrogen acceptor.

In an effort to localize the enzyme activity, Li and Bonner (58) and Wildman and Bonner (98) reported that it was not associated with chlorophyl nor with the chloroplasts, but is a readily soluble protein found in the cytoplasm. On the other hand, Arnon (3), as a result of his experiments, thought that the enzyme in chard leaves is located in the chloroplasts.

An extensive study of tyrosinase in strains of the fungus *Glomerella cingulata* was made by Markert (63) in order to discover the effects of genetic changes on the enzyme's activity. He concluded that the enzyme is under genetic control by a number of genes and that pigmentation can be similarly correlated.

TYROSINASE IN PIGMENTATION AND CUTICLE HARDENING

Pigmentation in melanoma tumors has been correlated with the presence of tyrosinase enzymes by Hogeboom and Adams (51) and by Greenstein (48). The latter author did not find any such enzyme in unpigmented tumors of related type. In normal tissues Charles and Rawles (29) found tyrosinase in the germs of red and black chicken feathers, but the enzyme was missing from the feather germs of white leghorns. Ginsberg (47) likewise made similar observations with guinea pigs; Danneel (33) described a dopa-phenolase-catecholase system in Drosophila; and Ohlmeyer and Hullstrung (72) found a possibly related enzyme in cases of the skin disease, vitiligo.

For other normal systems outside the plant kingdom Dennell (38) and Needham (70) have proposed a system, involved in the hardening of the cuticle of crustacea, which consists of a tyrosinase-polyphenol oxidase acting on such substrates as dopa at the time of moulting or limb regeneration. Dennell (39), as well as a number of other workers, observed the action of tyrosinase during the formation of the hard dark exocuticle in metamorphosing insects. Dennell correlated the activity of the enzyme with various stages of the life cycle. Heller (50), also working with insects, suggested that the tyrosinase present oxidizes substrates which are then reduced by cozymase as a part of the respiratory system.

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DISCUSSION

Dr. KAPLAN: I would like to ask if those preparations which seem to contain low copper content do have low catecholase activity and relatively high cresolase activity.

Dr. MALLETTE: The catecholase activity on a copper basis seems to be always a little lower, from one-third to one-half of what it is in those preparations which are considered to have a high catecholase activity.

Dr. KAPLAN: What happens if you add copper to your reaction?

Dr. MALLETTE: Whenever copper is added to tyrosinase preparations the activity increases a little, but it approaches maximum activity very rapidly. There is, therefore, some stimulation when copper is added to such preparations.

Dr. KAPLAN: Does it have any effect on the ratios of catecholase and cresolase activity?

Dr. MALLETTE: I cannot answer that question, since I do not know whether that experiment has been done.

Dr. Chance: Would you care to make any kind of general statement on the specificity of tyrosinase with special reference to Dr. Dawson's remarks this morning that it was the enediol structural grouping which was particularly suitable for this type of enzyme?

Dr. Mallette: Well, I am not exactly sure what you have in mind. I should have remarked that tyrosinase had no catalytic effect upon compounds that are derivatives of resorcinol or hydroquinone. Is that the answer you want?

Dr. Chance: Yes. However, have you completely removed inactivation effects? It may be that if you change the structure of the substrate you will alter the rate of inactivation.

Dr. Mallette: Well, I can't recall whether anyone has ever carried out any experiments in which they added the enzyme to resorcinol or to hydroquinone and then tested for activity afterwards. I suppose they may have. Do you know, Dr. Dawson?

Dr. C. R. Dawson: The enzyme will not catalyze the direct aerobic oxidation of hydroquinone or resorcinol. In a system containing enzyme, hydroquinone, and a small amount of catechol, oxygen is absorbed and the hydroquinone is oxidized. However, the oxidation undoubtedly proceeds via a shuttle mechanism involving the oxidation product of the catechol, i. e., the o-benzoquinone. The rate of oxidation is slower than that of catechol alone, and the hydroquinone appears to act as a competitive inhibitor rather than an inactivator. Only the catechol is oxidized in a tyrosinase-catechol-resorcinol system.

Dr. Mallette: Hydroquinone itself does not destroy the enzyme. I remember doing that myself, but with regard to resorcinol I can't tell you.

Dr. C. R. Dawson: Catechol or alkylated catechols have no effect on the enzyme as long as the enzyme does not function. In other words, if one incubates the enzyme with catechol in an anaerobic system and then exposes it to oxygen, there is no loss in activity towards catechol. Once the catalytic oxidation starts, however, enzyme inactivation is observed. The extent and speed of inactivation is markedly dependent upon the structure of the catechol. For example, 4, 5-dimethyl catechol is oxidized for a considerable length of time with very little evidence of enzyme inactivation. On the other hand, the enzymatic oxidation of 4-methyl catechol results in very rapid inactivation of the enzyme.

Dr. Chance: I was thinking of the work of Balls and Hale on a different type of enzyme, peroxidase, and some of their results have later been attributable to inactivation rather than real differences of specificity.

Dr. Mallette: The enzyme will catalytically oxidize phenol and p-cresol a little better in the presence of hydroquinone than in its absence.

Dr. GRANICK: What have you observed with respect to the rates of inactivation with these various substituted compounds?

Dr. C. R. DAWSON: We prepared the various substituted catechols such as 4-methyl catechol and 4, 5-dimethyl catechol, so as to compare their rate of enzymatic oxidation with the rate of enzyme inactivation. There was no simple correlation between the two rates. If the inactivation were due to a product of the reaction, such as o-quinone or hydrogen peroxide, a direct correlation between the two rates might be anticipated.

Dr. ARNON: Have you tried any other substitutions in the 3, 4 position?

Dr. C. R. DAWSON: Yes, we put a tertiary butyl group in the 4 position of catechol.

Dr. ARNON: How does this compare to catechol?

Dr. C. R. DAWSON: 4-tertiary butyl catechol oxidizes more slowly than catechol, and there is much less inactivation. The dimethyl catechol and 4-tertiary butyl catechol result in about the same inactivation, but the dimethyl catechol is oxidized more rapidly.

Dr. ARNON: But is the inactivation effect reduced?

Dr. C. R. DAWSON: No, the inactivation observed in the case of 4, 5-dimethyl catechol is about the same as that observed for 4-tertiary butyl catechol for a given initial rate of oxidation.

Dr. ARNON: The inactivation is of the same order of magnitude?

Dr. C. R. DAWSON: This depends upon the comparison that is being made. It takes more enzyme to give a certain initial rate of oxidation in the case of tertiary butyl catechol than it does in the case of 4, 5-dimethyl catechol, thus the inactivation per weight of enzyme is less in the t-butyl catechol case. When you carry this over into more complex structures such as dopa or chlorogenic acid, there is no simple correlation between substrate structure, enzyme, and amount of reaction inactivation. For example, the phenolase of the sweet potato is not inactivated very much when it acts on its natural substrate, chlorogenic acid, but is inactivated when oxidizing catechols. The mushroom enzyme is inactivated extremely rapidly when it acts on chlorogenic acid, even more rapidly than when oxidizing catechol. As pointed out before, the inactivation experiments on a series of substituted catechols were set up so that the oxidation would proceed at the same rate for each of the various compounds. Under these conditions one could say that there would be the same amount of o-quinone, or hydrogen peroxide, or a free radical formed in unit time in each case. It was found that there was no correlation between the rate of oxidation of these compounds per gram of enzyme used and the inactivation of the enzyme.

PHENOL OXIDASES AND PLANT RESPIRATION

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Based on the work of Bertrand and others on the oxidases, laccase and tyrosinase, the Russian botanist, Palladin (6), suggested that oxidases take part in plant respiration by catalyzing the aerobic oxidation of chromogens, usually phenolic bodies, which in turn are reduced by hydrogen donors farther back in the respiratory chain. This concept can be expressed today as follows:

$$\begin{tikzcd}[column sep=small] \text{Respiratory pigments (Quinones)} \arrow[d, shift right=3, "\text{Preceding part of the respiratory chain } 2H"'] \\ \text{Respiratory chromogens (Phenolic bodies)} \arrow[u, shift right=3, "O_2 \text{ and oxidases}"'] \end{tikzcd}$$

Among the first to try to demonstrate experimentally that polyphenol oxidase (tyrosinase) actually takes part in respiration were Boswell and Whiting (2). They added some catechol to respiring potato slices and observed a marked increase in the rate of oxygen uptake, accompanied by a small increase in the rate of carbon dioxide given off (Fig. 1).

Realizing that catechol might not be the natural hydrogen carrier, operating next to the terminal oxidase, they set out to isolate from potatoes the natural substrate. They were not able definitely to identify the product which they isolated, but it gave the green color with ferric chloride characteristic of ortho dihydric phenols. When the isolated substance, instead of catechol, was added to the respiring potato slices, more encouraging results were obtained. Both the increased rates of oxygen uptake and of carbon dioxide evolved continued above the normal rates of the slices for a considerable length of time, thereby indicating an increased rate of respiration (Fig. 2).

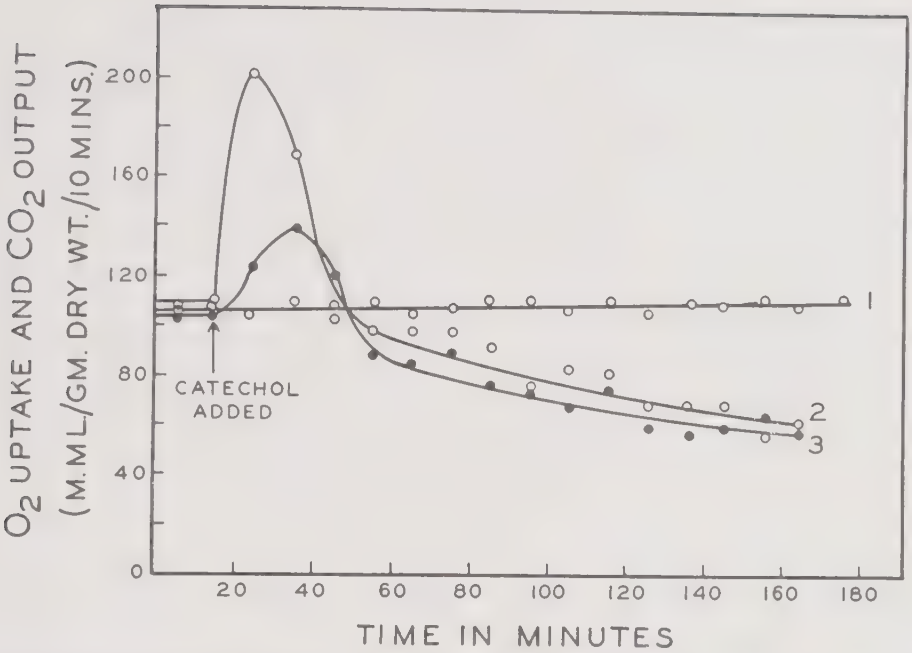


Fig. 1. The Rate of Respiration of Potato Slices When Catechol is Added

Horizontal axis, time in minutes. Vertical axis, O_2 and CO_2 in m. ml./g. dry wt./10 min. Curve 1, Phosphate alone; O_2 uptake. Curve 2, Phosphate and 0.5 ml. M/25 catechol; O_2 uptake. Curve 3, Phosphate and 0.5 ml. M/25 catechol; CO_2 output. From Boswell and Whiting (2).

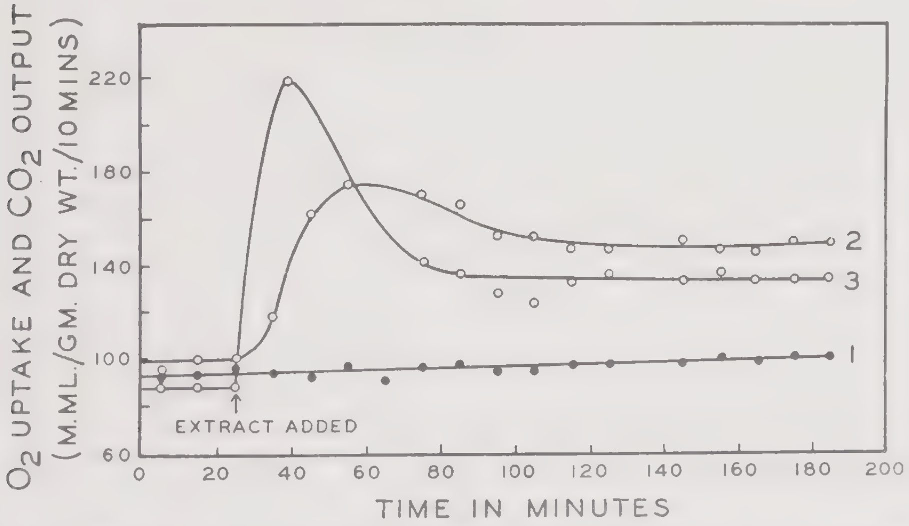


Fig. 2. The Rate of Respiration of Potato Slices Upon Addition of the Isolated Normal Substrate

Horizontal axis, time in minutes. Vertical axis, O_2 uptake and CO_2 output. m. ml./g. dry wt./10 min. Curve 1, Phosphate only; O_2 uptake. Curve 2, Phosphate and extract; O_2 uptake. Curve 3, Phosphate and extract; CO_2 output, bound CO_2 included only in the 30-50 min. period. From Boswell and Whiting (2).

It occurred to Baker (1) working in our laboratory, that possibly the less satisfactory results obtained when catechol was added to the respiring slices might be due to a too rapid oxidation of the catchol. As a result, the rest of the respiratory chain in the cells might not be able to keep the oxidation products continuously reduced as they were being formed. Such a condition would lead to an accumulation of the oxidation products which might be toxic, kill the cells, and stop respiration. With this idea in mind, Baker decided to add protocatechuic acid instead of catechol to the respiring potato slices. Protocatechuic acid is oxidized more slowly than catechol by potato tyrosinase. As can be seen in Fig. 3, both the rates of oxygen uptake and carbon dioxide given off were increased over those of the slices respiring alone, and these higher rates continued for some time.

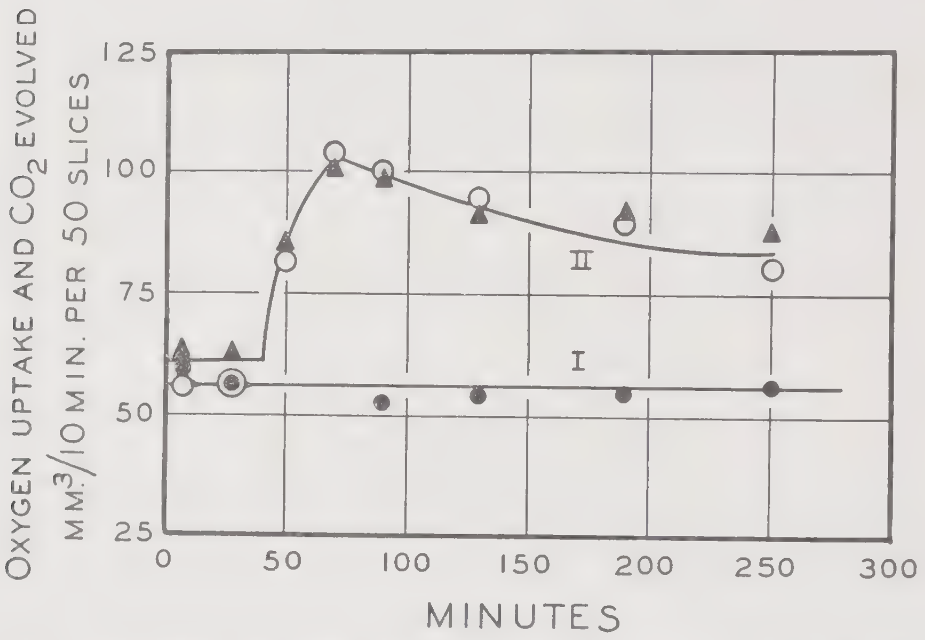


FIG. 3. INFLUENCE OF ADDED PROTOCATECHUIC ACID ON THE RATE OF RESPIRATION OF POTATO SLICES

Curve I, Control, shows the rate of O_2 uptake for 50 slices respiring in phosphate solution. Curve II, rate of O_2 uptake and CO_2 output when 2 mg. of protocatechuic acid were added 40 min. after the slices had been respiring in phosphate alone. Circles, O_2 uptake; triangles, CO_2 output. After Baker and Nelson (1).

Schade and coworkers (5) of the Overly Research Laboratories, permitted potato slices to respire in the presence of a mixture of 95% CO and 5% O_2, both in the dark and in the presence of strong light. They found that the respiration was retarded in the dark about 35 to 40 per cent, but when the slices were exposed to light, then practically no inhibition occurred. Since cytochrome oxidase, which they had shown to be present in the potatoes, is inhibited by carbon monoxide only in the dark, they concluded that 35 to 40 per cent of the respiration of the potato slices was dependent on cytochrome oxidase as the terminal oxidase. Furthermore, since tyrosinase action is inhibited by CO both in the dark and in light (4), they argued that the terminal oxidase serving for the remaining 60 to 65 per cent of the respiration of the slices could not be tyrosinase, but must be some other oxidase which is insensitive to carbon monoxide.

TABLE 1

Contents of Respirometer Flasks in ml.

Flask No.	27	26	28	29	30	31	1
Homogenate 20%	...	7.0	7.0	7.0	7.0	7.0	...
Hydroquinone soln. 2 mg. per ml.	...	...	...	...	1.0	1.0	1.0
Catechol soln. 10 mg. per ml.	...	...	0.5	0.5	...	...	...
Cytochrome C soln. 0.86 x 10^{-7} M	...	...	...	1.5	...	1.5	1.0
Water	10.2	3.0	2.5	1.0	2.0	0.5	8.0
20% KOH in wick	...	0.2	0.2	0.2	0.2	0.2	0.2
mm^3 of O_2 uptake in 80 minutes	...	28	124	120	−8*	52	−4*

* Exper. error.

Since respiring slices of some varieties of sweet potatoes also give an increase in oxygen uptake when catechol is added, Walter (11), in our laboratory, attempted to determine whether or not cytochrome oxidase might be functioning as a terminal oxidase. An homogenate from sweet potatoes was prepared and placed in a series of respirometer flasks together with cytochrome C, hydroquinone (which is not oxidized by the sweet potato oxidase), and catechol, as indicated in Table 1.

It will be observed from the data on the oxygen uptake given in the table that the amounts of oxygen absorbed by the catechol homogenate system (Flask 28), and the catechol-homogenate cytochrome C system (Flask 29) were about the same. In other words, the homogenate showed considerable catecholase activity, but the added cytochrome C caused no increase in the amount of oxygen uptake. The hydroquinone-homogenate-cytochrome C system (Flask 31) showed some activity, but the slight increase over that observed in the control flask (Flask 26), 24 mm^3, can hardly be attributed to the presence of an appreciable amount of cytochrome oxidase. The latter, therefore, did not seem to play any major role as a terminal oxidase in the respiration of the sweet potato slices.

Szent-Györgyi and Vietorisz (9) were impressed by the rapid and large amount of oxygen taken up when potato tissue was ground up and the cells injured, in comparison with the rate and amount of oxygen taken up when the potato tissue was permitted to respire in the form of slices. This large difference led them to conclude that the phenol oxidase (tyrosinase) and its substrate exist separately in the potatoes, and that oxidation only sets in when the cells are injured and the oxidase and the substrate are allowed to come together. According to this view, the potato oxidase does not serve as a respiratory enzyme, but more likely as a protective enzyme. When the plant issue is injured and oxidation sets in, the oxidation products formed, such as quinones, not only act in a bactericidal way, but also may exert a tannin action on the proteins present and thus form a protective covering for the injured part of the plant.

This idea that at least some of the phenol oxidases in plants exist in a dormant state has gained support from observations made by Appleman and coworkers (8), at the Maryland Experiment Station. They found that killing the cells in the sweet potatoes by heating above 60° C for a few minutes, or by treating them with alcohol or acetone, greatly increased the catecholase activity. Walter (10), at Columbia University, also noticed a large increase in the catecholase activity of sweet potato slices, if the cells of the latter were first injured by letting the slices remain in a 3 per cent saline solution for several hours (Fig. 4).

The Maryland Experiment Station investigators also noticed that the greatest discoloration on the cross section of a sweet potato root, when exposed to air, occurs in the primary and secondary areas of the cambium, areas which not only contain the greatest concentration of catecholase but also of catechol tannins. Kertesz (3), of the New York Agricultural Experimental Station, likewise found that the flesh of the peach (Sunbeam variety) does not discolor when exposed to air, due to the absence of catechol tannins in this variety of peaches.

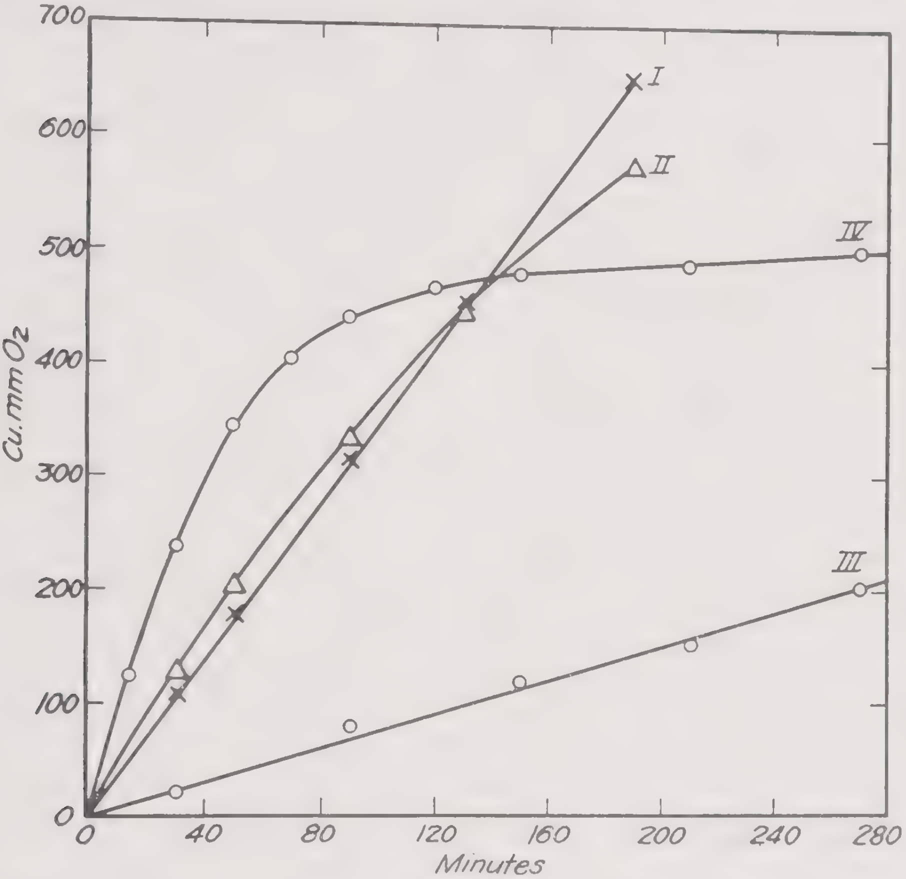
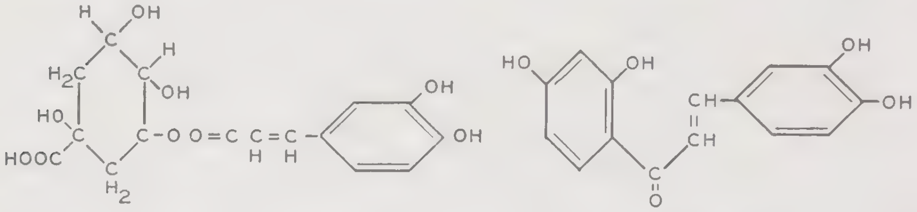


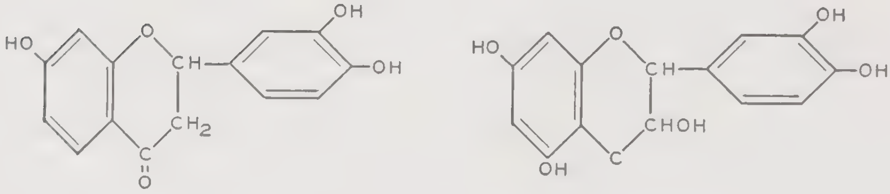
Fig. 4. The Effect of Catechol on the Rate of Oxygen Uptake of Yellow Jersey Sweet Potato Slices Washed in Saline Solution and Tap Water

Curve I, O_2 uptake of 30 slices washed 18 hrs. in running tap water. Curve II, same with addition of 10 mg. catechol at zero time. Curve III, O_2 uptake of 30 slices washed 3 hrs. in 3% NaCl solution, and then 18 hrs. in running tap water. Curve IV, same except 10 mg. catechol added at zero time. After Walter and Nelson (10).

In our laboratory, Rudkin (7), has isolated from sweet potatoes two substances, chlorogenic acid and component A, which show many of the characteristics of the catechol tannins. Although the chemical constitution of component A was not determined, it, like chlorogenic acid, gave a green color with ferric chloride and precipitated gelatin from aqueous solutions. Both yielded protocatechuic acid on alkaline hydrolysis, and component A gave the red phlobaphene reaction when boiled with mineral acids, a reaction characteristic of the catechol tannins.



CHLOROGENIC ACID CHALCONE



FLAVANONE CATECHIN

Fig. 5. Structure of Chlorogenic Acid and Related Compounds

The structure of chlorogenic acid and some related compounds are shown in Fig. 5. The fact that the quinic acid part of the chlorogenic acid molecule breaks down easily into phenolic compounds tempts one to suggest that the chlorogenic acid may, in the plant, serve as a forerunner of chalcones and finally of catechin and catechol tannins.

As can be seen from Figs. 6 and 7, both the chlorogenic acid and component A, when added to respiring sweet potato slices, appear not only to increase the rate of oxygen uptake but also the rate of carbon dioxide given off.

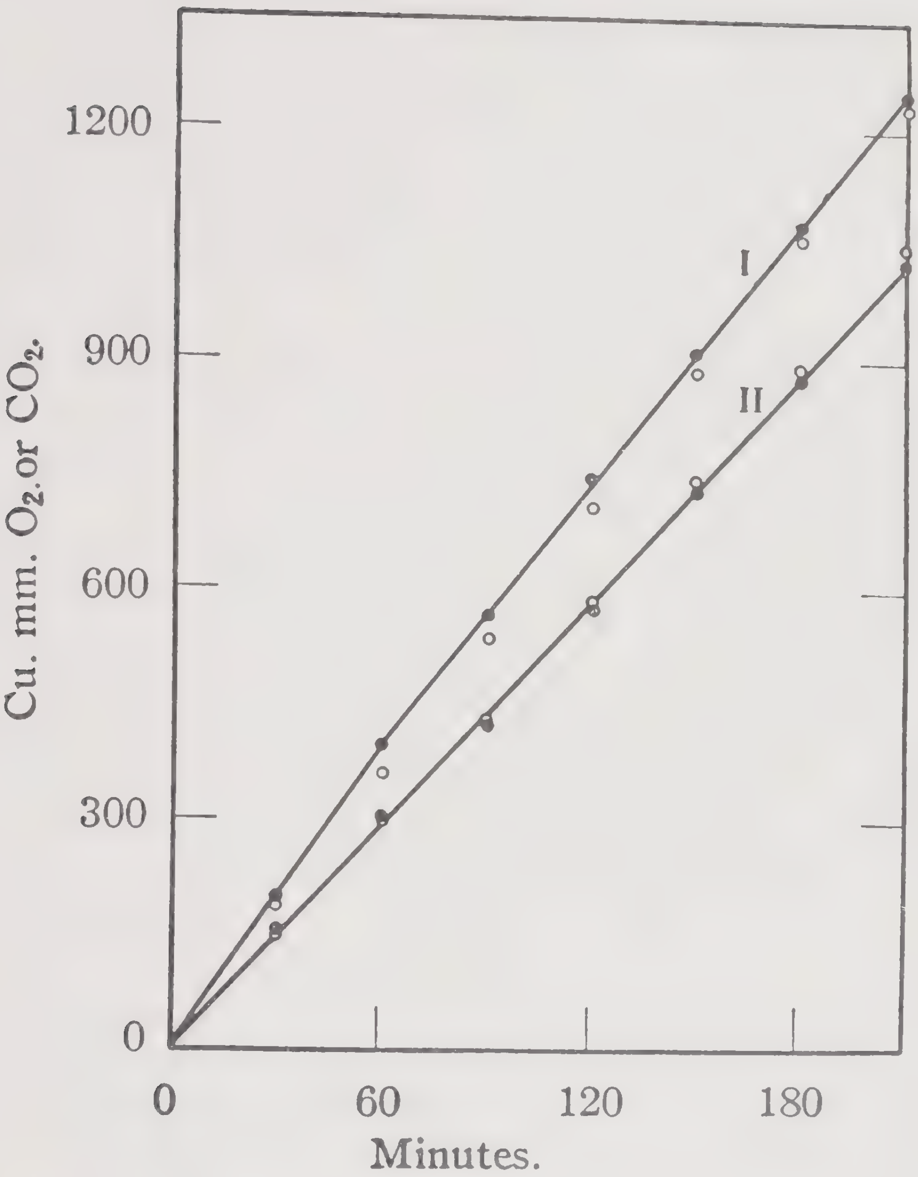


FIG 6. CURVE I: showing the increase in rates of oxygen uptake (solid circles) and carbon dioxide given off (circles) when 1 mg. of chlorogenic acid was added to thirty-five sweet potato slices respiring in a phosphate buffer solution, *p*H 5.5. CURVE II, control: rates of oxygen uptake (solid circles) and carbon dioxide given off by thirty-five sweet potato slices respiring in the absence of added chlorogenic acid.

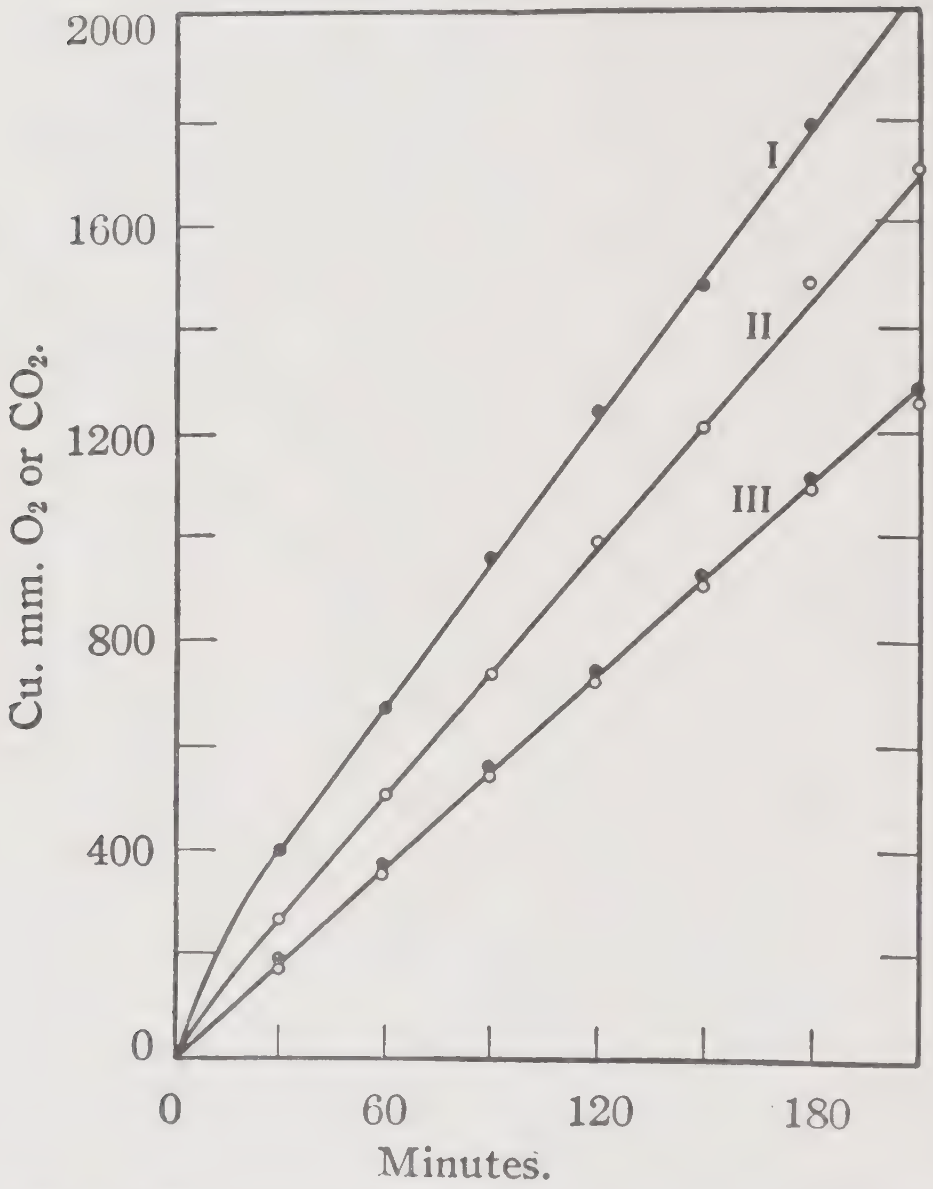


FIG 7. Curves I and II show the respective rates of oxygen uptake and carbon dioxide given off when 5 mg. of component 2 of component A was added to thirty-five respiring sweet potato slices. Curve III, control: rates of oxygen uptake (solid circles) and carbon dioxide given off (circles) by the respiring sweet potato slices without the addition of component 2 of component A.

Some experiments were also carried out in our laboratory by Walter (10) on the anaerobic respiration of slices of sweet potatoes, Irish potatoes, beets, and carrots, all of which had been previously washed for several hours in cold running tap water. It was found that addition of some ascorbic acid to such slices respiring anaerobically in an atmosphere of nitrogen kills the cells. If, however, potassium cyanide or sulphydryl compounds, such as cysteine or glutathione, are added along with the ascorbic acid, then no injury to the cells occurs (Fig. 8). The potassium cyanide and the sulphydryl compounds must have tied up or inactivated something in the slices which in the presence of the ascorbic acid became strongly toxic to the cells. Slices which had not been washed for any length of time in running tap water were not subject to this toxic action of the ascorbic acid when they were respiring anaerobically. It seems, therefore, that washing the slices in running tap water for several hours removes something which prevents the ascorbic acid, under anaerobic conditions, from exerting this toxic effect.

Potassium cyanide is usually thought of as an inhibitor of enzymes that contain a metal, such as copper or iron, enzymes which are usually not regarded as taking part in anaerobic respiration. The fact that cyanide can play this protective role against the ascorbic acid inactivation suggests that possibly some metal-bearing enzyme might be involved in anaerobic respiration or even in glycolysis.

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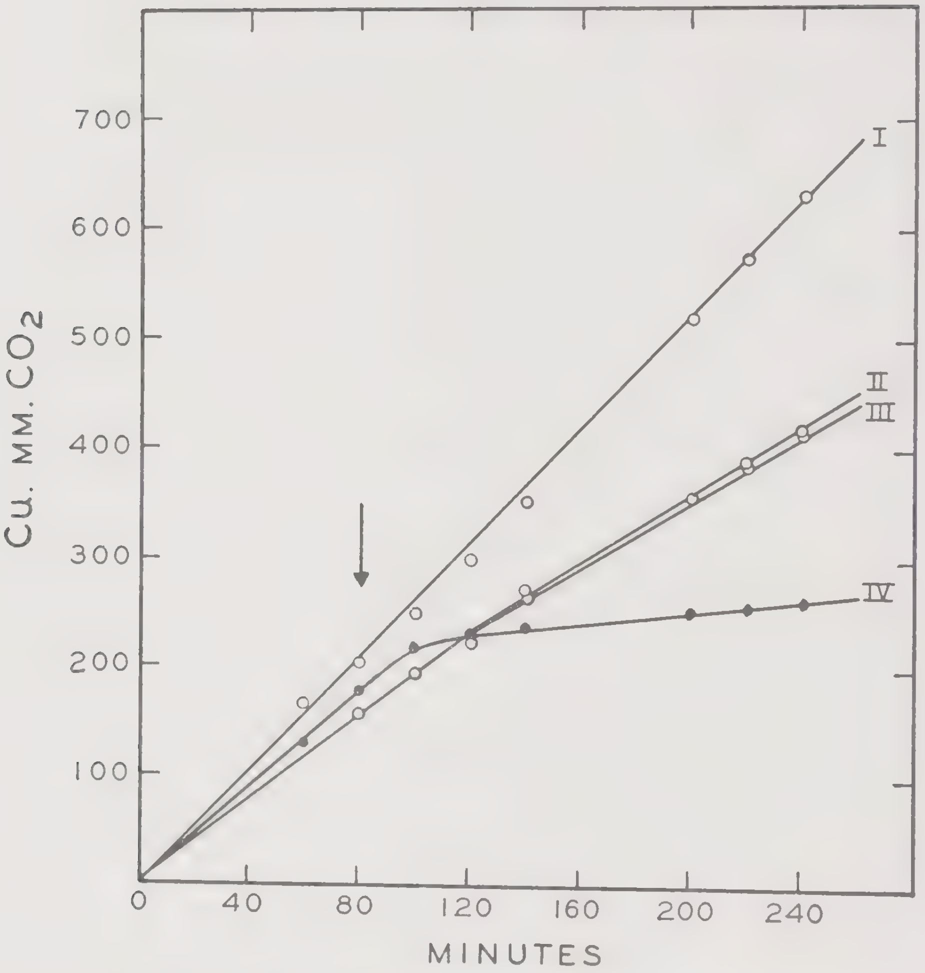

FIG. 8. Potassium cyanide preventing the inhibition of the anaerobic CO_2 production of sweet potato slices by added ascorbic acid. Thirty sweet potato slices, washed 20 hours in cold tap water, were placed in each respirometer flask together with a buffer solution in an atmosphere of nitrogen. (See experimental details). Curve I represents the rate of CO_2 given off by the slices respiring anaerobically in the buffer solution. Curve II represent the rate of CO_2 given off by the slices respiring in the presence of 5 mg. of KCN. Curve III is the rate of CO_2 given off by the slices in the presence of 5 mg. of KCN to which 3 mg. ascorbic acid were added from the side arm after the reaction had progressed for 80 minutes. Curve IV is the rate of CO_2 given off by the slices in the presence of 3 mg. ascorbic acid added after 80 minutes.

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DISCUSSION

DR. STEINBERG: Would you agree that these enzymes play a part in respiration?

DR. NELSON: No, I would not. Some evidence is strong for it, and some evidence points the other way. I think, therefore, that we would need more evidence before we can come to a definite conclusion.

DR. ARNON: It seems to me that there is no necessary conflict between the view that 1) tyrosinase may do certain things *in vitro* when the cell is broken, including a protective action such as you described; and that 2) in the cell these substances may occur in very low concentration and act catalytically in alternate oxidation reactions. This may certainly represent a difference in organization under these two conditions. When the cell is broken, the enzyme may come in contact with much higher concentrations of these quinone derivatives and would, therefore, produce products at a much greater rate and create a condition which would be toxic to the tissue. I think, therefore, we should not rule out the possibility that these enzymes function normally in this manner in the cell, but that this toxic effect is only observed when the tissue is destroyed. It should be pointed out in this regard that the enzyme can oxidize reduced coenzyme I and II as shown by Kubowitz. Potentially, therefore, we have a dual function for this enzyme.

DR. CHANCE: Would Dr. Nelson care to comment upon the possibility that cytochrome oxidase is a copper enzyme?

DR. NELSON: No, we have had no particular experience with this problem.

DR. CHANCE: But don't you think this is a related question?

DR. NELSON: That may be, but I have no information on the point. The Overly group thinks it is.

DR. KAPLAN: If you make a potato homogenate, and you add catechol and obtain a stimulation of oxygen uptake, do you also obtain an increased rate of carbon dioxide output?

DR. NELSON: I don't know. We just followed the oxygen uptake. I should say that we only get carbon dioxide output when we study live cells. When we kill the cells with ascorbic acid, carbon dioxide output is depressed immediately.

Dr. GRANICK: I would like to ask Dr. Britton Chance what he thinks about copper and cytochrome oxidase, since he seems to have a strong feeling about this.

Dr. CHANCE: I think it is a relevant question and warrants discussion.

Dr. GRANICK: The possibility of a copper enzyme coming into play in the cytochrome oxidase picture need not be dismissed summarily. Since one has to account for a transfer of 4 electrons from cytochrome C to D_2 to form $2H_2O$; and since such activity is yet known only in the heterogeneous mitochondrial material, it might be that a copper enzyme could cooperate with cytochrome oxidase in the process of oxidation with oxygen. Furthermore, if the energy released in cytochrome C oxidation is not to be wasted, it is necessary to postulate other enzyme interactions with this oxidation. The process of cytochrome oxidase reaction is, therefore, probably not to be regarded as a simple one-enzyme activity.

Dr. STEINBERG: Dr. Nelson, is it possible to replace copper in these enzymes with other heavy metals?

Dr. NELSON: No, not as far as I know. Kubowitz has tried to put in manganese, iron, and other trace elements but could restore no activity.

Dr. MALLETTE: Bodine tried the same thing with his protyrosinase and found that he could not replace copper.

FUNCTIONAL ASPECTS OF COPPER IN PLANTS

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INTRODUCTION

As with other micronutrients, the discovery of copper as an essential element for the growth of higher plants has occurred in advance of any knowledge of its function. In 1931, when Sommer first presented evidence for the indispensability of copper for higher plants, there was no knowledge of this metal participating in distinct cellular functions nor of its being a constituent of oxidizing enzymes.

Despite the great advances made in the last twenty years with regard to the biochemical functions of copper, a current discussion of its role in plant nutrition must take cognizance of the fact that it is still not possible to interpret many of its gross effects on plant growth and structure in precise biochemical terms. This review therefore will concern itself with the overall effect of copper on plant growth and physiological activity, as well as with the evidence bearing on the localization of the function of copper in the living cell through its being a constituent of specific enzymes or enzyme systems.

COPPER AS AN ESSENTIAL ELEMENT FOR HIGHER PLANTS

A clear demonstration of copper as an essential micronutrient for the growth of higher plants was first made by Sommer (40) for the tomato, sunflower, and flax. The onset of copper-deficiency symptoms was dependent on the removal of copper impurities from the nutrient medium. Thus in an earlier experiment, in which water distilled from a copper still was used, flax showed no symptoms of copper deficiency during the vegetative period of growth, but did when the fruiting stage was reached. When glass-distilled water was used, however, the same plant exhibited marked deficiency symptoms even during the early stages of growth (Sommer, 41). Lipman and MacKinney (30) presented evidence that barley plants were unable to produce seed without a supply of copper and drew the conclusion that copper is an essential element for plant growth. The numerous other experiments in nutrient solutions with different kinds of plants which confirmed the essentiality of copper have been reviewed elsewhere (Sommer, 41).

The early evidence for the need of copper by higher plants did not gain prompt acceptance for this element as a new essential micronutrient. It was suggested that the observed response to copper may have been due to an indirect influence, i. e., to its antagonistic or balancing effect on other elements (Raber, 35). Attention was also called to the desirability of confirming its specificity and of excluding the possibility that some other element capable of existing in several valence states could replace copper (Hoagland, 17). These considerations led Arnon and Stout (4) to re-examine the status of copper by testing its essential nature according to the following criteria: an element is not considered essential unless (a) a deficiency of it makes it impossible for the plant to complete the vegetative or reproductive stages of its life cycle; (b) such deficiency is specific to the element in question, and can be prevented or corrected only by supplying this element; and (c) the element is directly involved in the nutrition of the plant, quite apart from its possible effects in correcting some unfavorable microbiological or chemical condition of the nutrient medium.

Using the tomato as the test plant, it was found that copper satisfied these three criteria of essentiality. When the upper limit of copper impurity in the nutrient medium was reduced by means of appropriate techniques (Stout and Arnon, 45) to less than 0.0001 mg. to a plant, very severe, early deficiency symptoms were observed which, if uncorrected, led to death. A definite beneficial response was obtained from supplying 0.002 mg. of copper, but no effect was found from adding 20 additional elements which included those capable of existing in different valence states, such as molybdenum, vanadium, chromium, nickel, cobalt, titanium, and tungsten. In the minus-copper cultures the onset, type, and severity of copper-deficiency symptoms were the same whether these additional elements were supplied or not. When leaves of young tomato plants showing copper deficiency were sprayed with a dilute solution of $CuSO_4$ (0.02 p. p. m. of copper), recovery and resumption of normal growth of both shoots and roots took place (Fig. 1). These findings, showing the direct effect of copper on the plant as distinguished from its possible effects on the root environment, were interpreted as providing the final link in the chain of evidence of the essentiality of copper for plant growth.

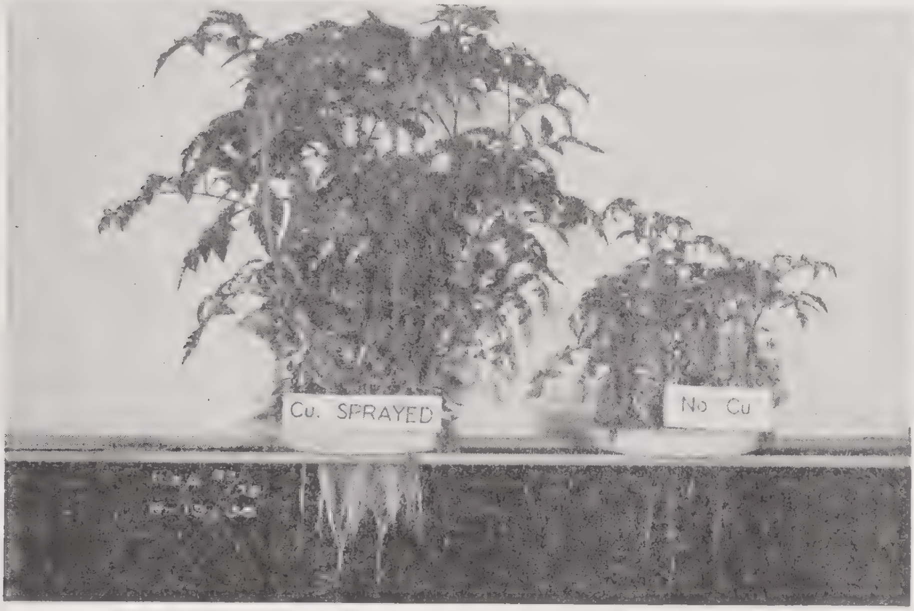


FIG. 1. Recovery of copper-deficient tomato plants following application of copper to leaves.

Copper deficiency symptoms do not constitute a uniform pattern in diverse species (Sommer, 41). In nutrient solutions, under controlled conditions, a consistently reproducible and distinct pattern is observed for a given species such as tomato, namely, very much stunted growth of shoots and roots, curling of leaves, failure to produce flowers and a bluish-green color of the foliage with a sheen very distinct from any other deficiency symptoms yet observed in that plant (Arnon and Stout, 4; Sommer, 41). A detailed study of the relation of copper deficiency to leaf structure was made by Reed (36).

EFFECT OF A VARIABLE COPPER SUPPLY ON PLANTS

The addition of copper to the list of essential micronutrients implies, of course, that no plant growth is possible without a supply of this element. As with most micronutrients, however, due caution has to be exercised in providing a concentration in the nutrient medium compatible with good growth, yet avoiding a toxic level. Thus for tomato in purified nutrient solutions 0.02 p. p. m. was found to supply adequately the copper requirement, but 2.0 p. p. m. was distinctly toxic (Arnon and Stout, 4). The sensitivity of the plant to this rather low concentration in the nutrient medium adds interest to the search for the full explanation of the function of copper in the metabolism of the plant.

Leaving the rather familiar ground of experimentally induced copper deficiency or toxicity, it is necessary to approach the still uncharted terrain of the copper requirements of plants. There are several lines of evidence which suggest that certain physiological activities and structural characteristics of plants are favorably affected by a modified supply of copper. Knott (23) found that on many areas of muck soils in New York State, onions frequently are characterized by poor color and thin scales, which have a tendency to come off in the handling incident to harvesting, grading, and sacking. This results in susceptibility to bruising and poor appearance, and leads to serious economic losses. Thicker scales and good color development were obtained from applications of copper sulfate to the soil. The improvement from adding copper to the soil was confined to quality, since affected areas produced onions of adequate size and yield. The beneficial effect of copper was not limited, however, to that element only; phosphorus alone was also effective in correcting the poor color and thin scales of onions grown on these muck soils.

Mulder (31) found that the external copper supply had a great influence on the tyrosinase activity of potato tubers. Tubers grown in soils poor in copper had a tyrosinase activity of less than one-tenth of that in tubers in comparable plots fertilized with copper sulfate. This effect of low copper on reducing the tyrosinase content was also found to be associated with the development of blue-black discoloration, which often appears in potatoes grown in soils low in potassium. Potassium deficiency seems to be associated with a high tyrosine content, which upon oxidation by tyronsinase gives rise to dark oxidation products of the melanin type. Thus on soils low in potassium, a low copper supply was beneficial in reducing the amount of blackening in potatoes. This type of blackening is to be distinguished from the blackening sometimes observed in potatoes after cooking.

In comparing the relative merits of ammonium and nitrate ions as the sole source of nitrogen for barley plants, it was found (Arnon, 1) that copper is one of the metals which can give a striking improvement in the growth of plants on an ammonium source. An additional supply of copper (0.05 p. p. m.) gave only a relatively minor improvement in the growth of plants supplied with nitrate nitrogen, but was associated with a four-fold increase in the dry weight of shoots and an eight-fold increase in the dry weight of roots of plants supplied with ammonium nitrogen. The effect of added copper was particularly striking when accompanied by supplemental aeration. Ammonium plants receiving the combination of copper and aeration were comparable in growth to the nitrate plants. The marked influence of copper on the utilization of ammonium nitrogen by barley was not specific. A similar effect was observed by adding other metals, notably manganese. It has been suggested (Arnon, 1) that the marked effect of added copper, manganese, and other metals, as well as of aeration on the utilization of ammonium nitrogen may be due, at least in part, to a compensating effect that these agents exercise on plants deprived of nitrate nitrogen. According to this view, the better growth which is usually obtained with most plants when nitrate ions are given as a source of nitrogen may be associated with the fact that nitrate is reduced in the plant to amino nitrogen. Furnishing the plant with nitrogen exclusively in the form of ammonium may thus be regarded as not merely a substitution of one source of nitrogen for another, but also as a deprivation of an oxidizing agent. The beneficial effect on ammonium cultures of added copper or of an increased oxygen supply is conceivably linked with the fact that these modifications of the ammonium culture medium tend to compensate the plant for the oxidative function of nitrate. Further elaboration of this theory must await appropriate biochemical studies.

PARTICIPATION OF COPPER IN THE METABOLISM OF GREEN PLANTS

The status of copper as an essential micronutrient and the explicitly small quantity in which it is required by plants suggested at once a catalytic function for it within the cell. Modern research in biochemistry has indeed established the role of copper as the prosthetic group in such metalloprotein, oxidizing enzymes as polyphenol oxidase (tyrosinase) and ascorbic acid oxidase. This constitutes prima facie evidence for the role of copper in those cellular processes in which these enzymes are known to participate. To the extent, therefore, that copper oxidases are shown to be essential for the respiration of a given species, the role of copper in that process is obvious. The part which copper oxidases play in respiration will be reviewed in a later section.

It appears certain, however, that in our present state of knowledge it is not possible to confine a discussion of the physiological role of copper to the copper enzymes known today. The catalytic effect of copper in cellular reactions may express itself through the activity of enzymes not yet discovered or perhaps even in some other manner, which cannot as yet be clearly envisaged. Example in support of this point of view may be cited from both animal and plant physiology. It is still not possible to define the role of copper in hemoglobin formation in terms of distinct enzymatic reactions (Elvehjem, 15). Nor is it possible as yet to link the known copper enzymes to the reactions of photosynthesis, despite indications that this metal may be involved in the photochemical carbon dioxide fixation by green cells.

The case for the function of copper in photosynthesis must rest as yet on rather indirect evidence. Neish (32) found that in the clover leaf copper is concentrated in the chloroplasts: 74.6 per cent of the total copper in the leaf was localized in the chloroplasts and, significantly, most of it was found to be in organic combination. Iron, by comparison, was also concentrated in the chloroplasts, but although an appreciable portion of it was in organic combination, the major part was in an inorganic form (soluble in 10 per cent acetic acid). Green, McCarthy, and King (16) found that several inhibitors such as thiourea, salicylaldoxime, and sodium diethyl dithiocarbamate, which are generally regarded as poisonous for copper enzymes, inhibit both photosynthesis and respiration of *Chlorella*, photosynthesis being the more sensitive to the action of the inhibitors. The inhibition was removed by dialysis.

Li and Bonner (29) reported that in tea leaves, polyphenol oxidase was associated with grana, and Arnon (2) presented evidence that in chard leaves polyphenol oxidase is localized in the chloroplasts. Unpublished data by the author have led to the same conclusion for sugar beet leaves. The concentration of a copper-protein oxidase in a cellular body so intimately identified with the process of photosynthesis raises the question of the possible participation of this enzyme directly or indirectly in one or more of the biochemical reactions linked with photosynthesis. The answer to this question must await further experimentation. It is of interest, however, that in the photolysis of water by isolated chloroplasts, quinones can serve as hydrogen acceptors (Warburg and Lüttgens, 51; Aronoff, 7; Arnon and Whatley, 5). Quinones are known as products of the enzymatic activity of polyphenol oxidase. What the natural primary acceptor of hydrogen is in photosynthesis (as distinguished from the ultimate one, which is carbon dioxide) is as yet not known. Although no suitable mechanism can be proposed at this time, it is perhaps permissible to speculate that should a quinone-like substance prove to be the natural hydrogen acceptor in green cells, a copper enzyme such as polyphenol oxidase may be responsible for its oxidation from a corresponding phenolic compound. However, the legitimacy of assigning a universal function to polyphenol oxidase in photosynthesis is linked with the question of its distribution in plants. This point will be discussed in a later section.

Recent unpublished experiments in the writer's laboratory (Arnon and Whatley, 6) lend support to a hypothesis that metals take part in the light reaction of photosynthesis, as measured by the evolution of oxygen by isolated chloroplasts. This reaction is strongly inhibited by several inhibitors known to inactivate metal protein enzymes. Orthophenanthroline and potassium ethyl xanthate were found to be particularly suitable. Other metal inhibitors such as 2, 3-dimercaptopropanol (BAL), cysteine, sodium, or diethyldithiocarbamate, while also giving strong inhibition, were inappropriate, since experimental analyses of their inhibitory action disclosed their interaction with the oxidant used as the hydrogen acceptor.

Preliminary mention of the results of phenanthroline inhibition was made elsewhere (Arnon, 2). The powerful action of this inhibitor was reversed by the addition of several metals, among which were nickel and cobalt, not known to be essential for plant life. Reactivation was also obtained with copper and zinc, two essential micronutrients. The place of iron in the reversal of the phenanthroline inhibition is particularly noteworthy. In the previous report (2), mention was made of the finding that ferrous iron was only partly effective in reversing the phenanthroline inhibition; its action was less effective than that of either zinc, copper, cobalt, or nickel. Warburg and Lüttgens (51) had also previously reported that iron was less effective than zinc in reversing the phenanthroline inhibition of isolated chloroplasts. The relative ineffectiveness of iron was rather puzzling, in view of the fact that ferrous iron forms a strong complex with phenanthroline, characterized by a dissociation constant of 5×10^{-22} at 25°C (Lee, Kolthoff, and Leussing, 27). These facts suggested that the reversal of phenanthroline inhibition could not be wholly explained as a binding of the inhibitor by metals but had perhaps some other specific physiological basis.

Recently obtained evidence (Arnon and Whatley, 6), however, accounts for the previously observed anomalous behavior of iron. It was found that the reason why iron is rather ineffective in reversing the phenanthroline inhibition is that under the conditions prevailing during the experiment ferrous was oxidized to ferric iron, and the later is far less effective in binding phenanthroline (Fig. 2b). When conditions were so arranged that the oxidation of ferrous ion was prevented, iron was effective in reversing the phenanthroline inhibition of the photochemical oxygen evolution by chloroplasts (Fig. 2a). It now becomes permissible, therefore, to postulate that the reversal of the phenanthroline inhibition by several metals is caused by their complexing action with the inhibitor. The chemical rather than physiological properties of the metals are involved.

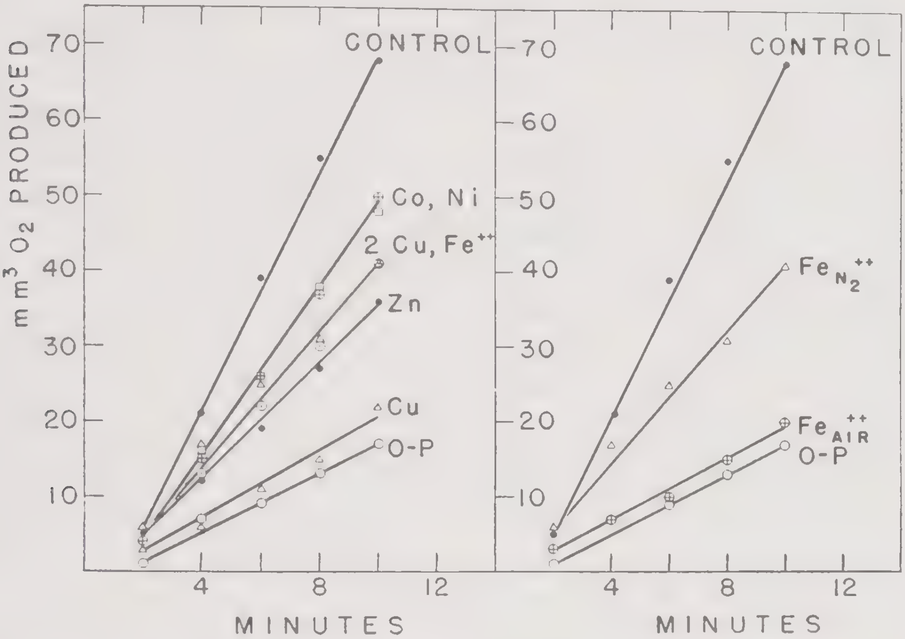


FIG. 2. Left (a) Effect of metals in reversing o-phenanthroline inhibition. 2×10^{-5}M o-phenanthroline and $2/3 \times 10^{-5}$M of the respective divalent metal salt.

Right (b) Reversal of o-phenanthroline inhibition by ferrous iron. Concentrations as in 2 (a).

It is still necessary, however, to account for the powerful inhibition by phenanthroline of the light reaction of photosynthesis, in the first instance. An adequate hypothesis must take cognizance not only of the previously described reversibility of the inhibition by metals, but also of another recent finding. It was observed (6) that the phenanthroline inhibition of chloroplasts was reversed by dialysis (Fig. 3a). We are dealing then with a powerful inhibition of an enzymatic reaction by a metal binding agent, but the inhibition is readily removed either by dialysis or by combination of the inhibitor with an externally supplied metal. We believe that these facts are explicable by a hypothesis that phenanthroline inhibits because it blocks the reactivity of an organic metal complex which is essential for the light reaction of the chloroplasts. The metal must be held very tightly in the organic complex, since it is not removed by dialysis against phenanthroline. It is interesting to recall in this connection the previously mentioned copper and iron organic compounds in the chloroplasts (Neish, 32). The inhibitor is assumed to enter only into a relatively loose combination with the metal in the organic

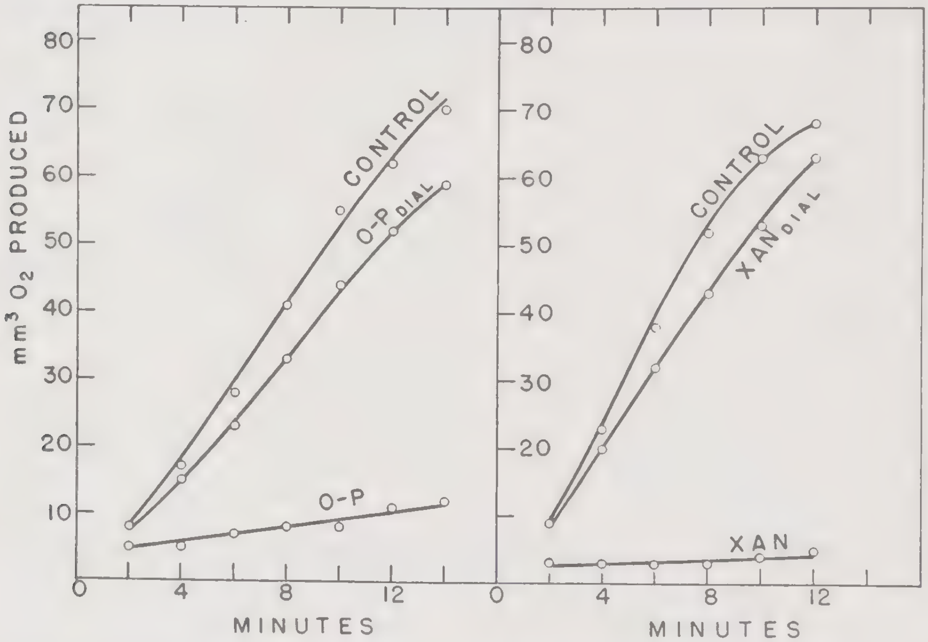


FIG. 3. Left (a) Reversal of o-phenanthroline inhibition by dialysis. 5×10^{-5}M o-phenanthroline. Dialyzed in the cold, overnight, against M/15 potassium phosphate buffer pH 6.5, containing 15% methanol.
Right (b) Reversal of potassium ethyl xanthate inhibition by dialysis. 6×10^{-3}M xanthate. Dialyzed in the cold, overnight, against 1% KCl solution containing 15% methanol.

metal complex. Dialysis or an external supply of a metal reverses the inhibition.

These considerations will be more fully developed elsewhere. What is pertinent to the present discussion is the identity of the metal in the postulated organic metal complex. In this connection it is of interest to review our recent findings with potassium ethyl xanthate (XAN), an inhibitor commonly regarded as fairly specific for copper enzymes (Green, McCarthy, and King, 16). As shown in Figs. 3b and 4, XAN was found to be a strong inhibitor of the light reaction of photosynthesis. The inhibition was reversible either by dialysis or by the addition of copper. The nature of XAN inhibition parallels that of phenanthroline, except that among the metals tried only *copper* is effective in reversing the inhibition.

It is considered premature to designate the metal which in all likelihood is involved in the light reaction of photosynthesis. This problem is under active study at the present time. Our current data, however, would permit us to say that the possibility cannot be excluded at this time that copper may prove to be at least one of the metals concerned in the light reaction of photosynthesis in green plants.

COPPER ENZYMES IN PLANTS

Laccase. In 1897 Bertrand, in France, reported that manganese was consistently associated with the activity in plants of an oxidizing enzyme, laccase. This announcement linked for the first time a metal with an oxidizing enzyme in living cells. It is true that recent work has shown that the effective metal in laccase is copper rather than manganese (Keilin and Mann, 22; Tissières, 48, 49), but the principle first proposed by Bertrand, that a metal forms the prosthetic group of an enzyme has retained its force and proved to be of outstanding significance in biochemistry.

Laccase differs from polyphenol oxidase in that it acts on p-phenylenediamine and is not poisoned by carbon monoxide. The crude enzyme also oxidizes ascorbic acid directly (Keilin and Mann, 22). The enzyme is found in the latex of several species of lacquer trees. Its function in the metabolism of the plant is as yet unexplained.

Enzyme inactivating indoleacetic acid. The regulation of the amount of growth substances in the plant is of considerable physiological importance. Tang and Bonner (47) found an enzyme in pea-seedlings capable of destroying indoleacetic acid. They found that the enzyme was inhibited by carbon monoxide but that the

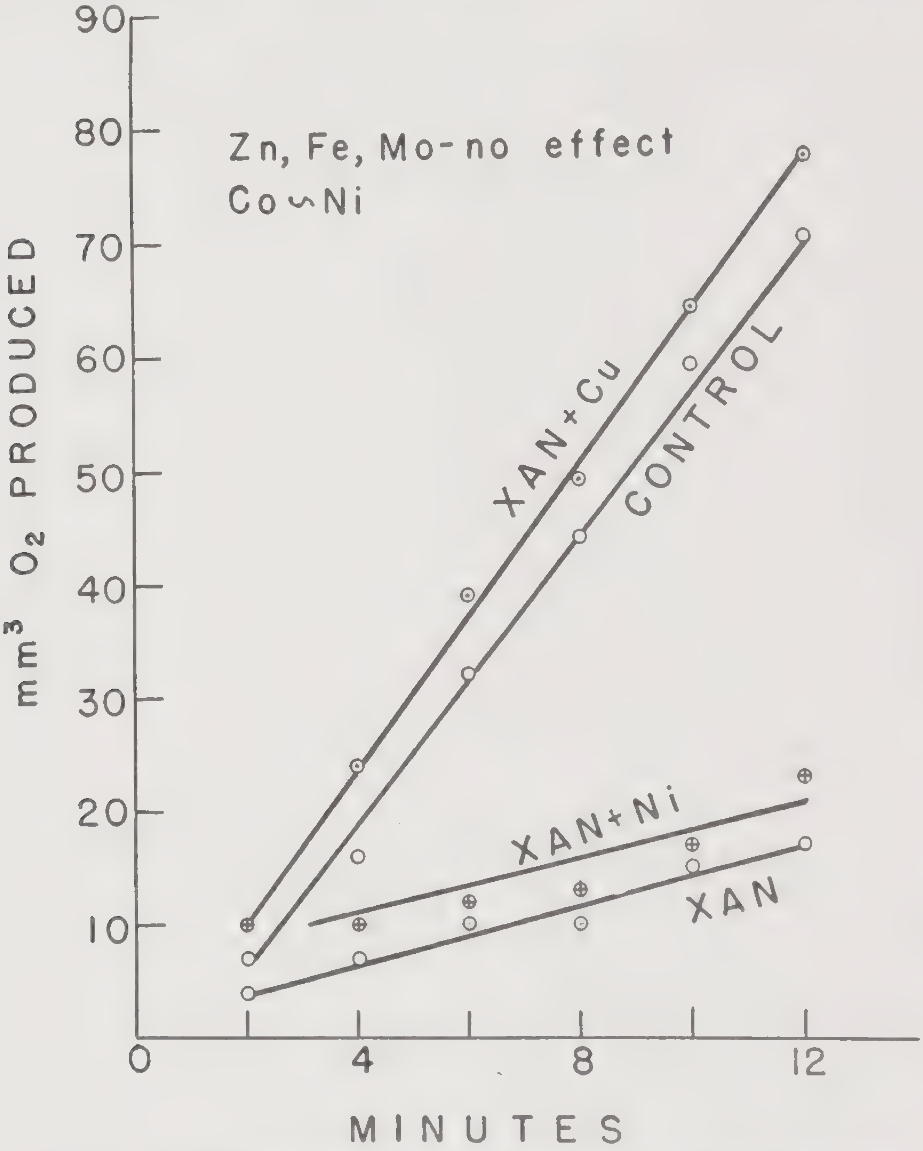


FIG. 4. Xanthate inhibition and its reversal by copper and other metals. 5×10^{-4}M Xanthate and 2.5×10^{-4}M of the respective metal.

inhibition was reversed by light. On the basis of these findings, Tang and Bonner were inclined to regard this enzyme as an iron protein, probably a heme-containing enzyme. These results, however, were not confirmed by the recent studies of Wagenknecht and Burris (50). The latter have presented evidence strongly suggestive of a copper-protein nature of the indoleacetic-inactivating enzyme found in bean roots and pea seedlings.

Ascorbic acid oxidase. The evidence in support of the existence of a definite ascorbic acid oxidizing enzyme in plants, as distinguished from a catalytic oxidation by copper linked to non-specific proteins, was comprehensively reviewed by Dawson and Mallette (13). In a recent comparison of the catalytic action of ascorbic acid oxidase and cupric ions on the oxidation of L-ascorbic acid and a series of compounds with structural similarities, Dodds (14) found that the enzyme catalyzes the oxidation of the dienol group, but only in those compounds in which this group is adjacent to a carbonyl group and ring structure. Ascorbic acid oxidase was further characterized by oxidizing the compounds of the ascorbic acid series only to the reversible or dehydro form, giving an oxygen uptake of one atom per molecule of substrate. By contrast, copper with the same substrates is a specific catalyst for the dienol group as such. In catalyzing the oxidation of the ascorbic acid series, copper causes the oxygen uptake to be greater than that required for oxidation to the reversible or the dehydro stage.

The extensive literature on the role of ascorbic acid oxidase in plant metabolism has been reviewed by James (19) and Waygood (52). The most obvious function of the enzyme is to be sought in plant respiration in its mediation of the electron transfer between reduced ascorbic acid and atmospheric oxygen. In this capacity as a terminal oxidase, ascorbic acid oxidase would be analogous to cytochrome oxidase, and ascorbic acid itself would correspond to cytochrome C, undergoing reversible oxidation and reduction.

It seems pertinent, however, to record some differences between the cytochrome system as it is known in animal tissues and in yeast and the ascorbic acid system in plants. Unlike cytochrome C, ascorbic acid can be oxidized not by one specific oxidase but by several enzyme systems known to occur in plants, not to mention the nonenzymatic catalysis by cupric ions. Polyphenol oxidase (Keilin and Mann, 21), peroxidase in the presence of orthodihydroxy compounds (Huszak, 19), the already cited crude laccase (Keilin and Mann, 22) as well as the cytochrome system (Keilin and Hartree, 20; Stotz et al., 44), have all been found to oxidize ascorbic acid. Moreover, although ascorbic acid seems to be universally distributed in plants, the same cannot be said about ascorbic acid oxidase. These observations are made not with the intent of prejudicing the case against the parallelism between ascorbic acid and the cytochrome system; indeed the very multiplicity of mechanisms for its oxidation in plants may be used as an argument in support of the role of ascorbic acid as a hydrogen carrier. It appears, however, that the problem has special ramifications in plants, and no ready analogy is available from other tissues.

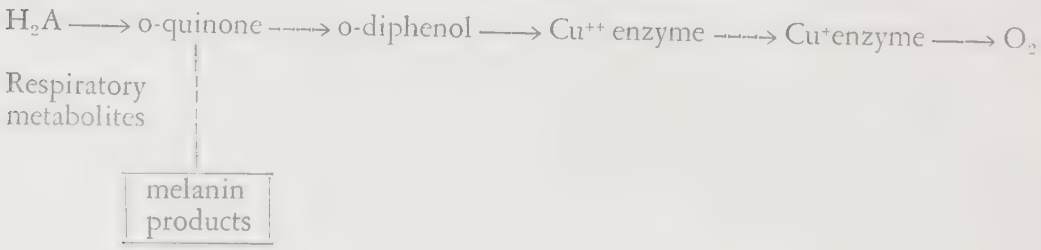
Until recently the main obstacle to the acceptance of ascorbic acid as a hydrogen or electron carrier in plant respiration has been the lack of a mechanism for the reduction of the oxidized ascorbic acid (dehydroascorbic acid). It was shown (Crook, 12) that extracts of many plants can catalyze the reduction of dehydroascorbic acid by glutathione, but the relation of glutathione to respiration in plants is not clear. The first evidence of physiological importance bearing on this problem was brought to light by James (19) and his coworkers. It was found that barley sap, which is characterized by the presence of ascorbic acid oxidase, oxidizes lactic to pyruvic acid by means of dehydroascorbic acid. The presence of lactate maintained the ascorbic acid in the reduced form; oxygen was being consumed at the expense of lactate, which was oxidized to pyruvate. Waygood (52) has recently proposed a scheme providing for oxidation and reduction of ascorbic acid in wheat through linkage to malic dehydrogenase and coenzyme I, with a flavin enzyme and a special pigment acting as intermediaries.

Polyphenol oxidase (*Tyrosinase*). Of all the copper enzymes in plants this enzyme has attracted the greatest interest. There exists a wide divergence of views about the significance of this oxidase in the metabolism of plant tissues, and a survey of the literature reveals that recent work has, in some instances, not only failed to resolve previous controversies but has indeed revived them. The extensive literature on the chemistry, mode of action, and role of polyphenol oxidase (also known as tyrosinase) has been recently reviewed elsewhere (Nelson and Dawson, 33; Dawson and Mallette, 13). The purpose of the present discussion is to examine certain salient features of what appears to be the present status of polyphenol oxidase in plant metabolism.

Since the enzyme catalyzes the aerobic oxidation of a variety of phenols, especially those characterized by an ortho-dihydroxy grouping, it was only natural to assign to this enzyme the place of a terminal oxidase in respiration (Onslow, 34; Boswell and Whiting, 11). The substrates which have received great prominence in discussions of the function of this enzyme are catechol and its oxidation product orthoquinone. Doubts have arisen, however, concerning catechol as a substrate for polyphenol oxidase (Szent-Györgyi, 46). The oxidation of catechol by polyphenol oxidase is extremely rapid, and no such vigorous oxygen uptake is found in respiration of plant tissues in which the enzyme occurs. Moreover the oxidation product, o-quinone, is a very reactive oxidant which, depending on its concentration, can be very damaging to living tissues. These difficulties led Szent-Györgyi (46) to discount the role of polyphenol oxidase in plant respiration and to assign to this enzyme a dormant status in normal tissues. According to this view, only when tissues are damaged, as by slicing or pathogenic invasion, is the enzyme mobilized to activity and led to produce the quinone which is toxic to the invader. The dark discoloration observed in damaged tissues was explained by the subsequent reactions of the quinone with amino acids and proteins, reactions giving rise to melanin-like substances.

Schade et al. (39) have very recently revived the argument against the view that polyphenol oxidase acts as a terminal oxidase for potato slices on the ground that the original evidence was secured with the use of catechol as a respiratory substrate (Boswell and Whiting, 11). The fact that catechol acts primarily as a cell poison in potato slices invalidates, in the opinion of Schade et al. (39) the view that polyphenol oxidase is involved in the respiration of these tissues. In a subsequent paper, Levy and Schade (28) have proposed alternative pathways for enzymatic terminal oxidations in the potato tuber.

As for the unsuitability of catechol as a substrate, this argument does not seem to weaken materially the case for polyphenol oxidase. Although catechol itself appears to be unsuited, other substituted phenols such a protocatechuic acid (Baker and Nelson, 8) and dihydroxyphenylalanine (dopa) (Robinson and Nelson, 37) are oxidized by the enzyme without complications. They are utilized without any untoward effects, such as discoloration of tissue, and this suggests that in the normal course of events their respective quinone oxidation products are reduced by the metabolites of the cell. In this manner these phenolic substances can act as electron or hydrogen carriers between the products of normal respiratory metabolism and atmospheric oxygen. As long as the normal path of metabolism remains undisturbed, these carriers would remain largely in the reduced state: as soon as their respective quinones were formed, they would be reduced by the respiratory metabolites. When the normal path of respiration is disturbed through injury the quinones would accumulate, and by subsequent reactions give rise to melanin-like bodies and bring about discoloration of tissue. On the basis of such considerations, Robinson and Nelson (37) proposed that in potato respiration the normal phenolic hydrogen carrier is dihydroxyphenylalanine (dopa). Dopa, which itself is derived from the irreversible oxidation of tyrosine, can undergo reversible oxidation to the ortho-quinone by the enzyme and in this manner acts as the electron carrier proper. Only small amounts of dopa are required for its effective function. The chain reactions can be represented schematically in the following manner, the solid arrows indicating the direction of electron transfer:



The reactivity of the enzyme with orthophenols other than catechol would seem to dispose of the objections to the view that the latter serves as a substrate. There are also other weighty considerations which endow polyphenol oxidase with great significance in the metabolism of plants. The enzyme, even when highly purified, can in the presence of a small amount of an orthodiphenol oxidize many substances of great physiological importance in the cell. Kubowitz (24, 25) has shown that polyphenol oxidase can, with the aid of 0.01 mg. catechol, oxidize reduced coenzyme I and in this way be linked to alcohol dehydrogenase. Polyphenol oxidase plus catechol can also oxidize reduced coenzyme II. This was shown by Kubowitz (26) with the use of glucose-6-phosphate dehydrogenase (Zwischenferment), which oxidizes glucose-6-phosphate to phosphogluconic acid. Keilin and Mann (21)) have demonstrated that in the presence of catalytic amounts of catechol (0.01 mg.), polyphenol oxidase oxidizes ascorbic acid.

These examples provide mechanisms for linking this enzyme to important pathways of cellular metabolism. In the examples cited the orthodiphenol, catechol, was used only in minute amounts as an electron carrier or "shuttle"; the substrates which were consumed were respectively alcohol, hexose phosphate, and ascorbic acid. Polyphenol oxidase can thus conceivably function as a terminal oxidase in the cell, even when its activity is linked with catechol. Because of the minute amounts in which catechol is required for these coupled oxidations, it could, despite its toxicity at higher concentrations, still be used without harm by the cell. Of special significance is the oxidation of the reduced coenzymes I and II, for in this manner the polyphenol oxidase system is joined to the principal oxidative pathways in the cell in which the various dehydrogenases act through the intermediary of either coenzyme I or coenzyme II. The chain of reactions linking polyphenol oxidase to the oxidative pathways in the cell can be represented schematically in the following manner, the solid arrows indicating the direction of electron transfer:

$$\begin{tikzcd}
 & & \text{CoI} \arrow[r, dashed] & \text{CoI } H_2 \arrow[dr] & & & \\
H_2A \arrow[r] & \text{dehydrogenases} \arrow[ur] \arrow[dr] & & & \text{o-quinone} \arrow[r, dashed] & \text{o-phenol} \arrow[r] & Cu^{++} \text{ enzyme} \arrow[d, dashed] \\
\text{substrates} & & \text{CoII} \arrow[r, dashed] & \text{CoII}H_2 \arrow[ur] & & & Cu^{+} \text{ enzyme} \arrow[d] \\
 & & & & & & O_2
\end{tikzcd}$$

Among the prominent arguments against assigning to polyphenol oxidase a universal function in plant respiration is the fact that the enzyme has been reported to be present only in about half of the many species examined (Onslow, 34). This argument, however, would not militate against the function of the enzyme in the species in which it is known to occur. As pointed out by James (19), there is evidence that the cytochrome system is not operative in all plant tissues or even in one tissue at different stages of growth, but these findings lead to no doubt concerning the role of cytochrome in respiration. Moreover, it must be borne in mind that most of the studies on the distribution of polyphenol oxidase in higher plants were carried out a long time ago, without the use of modern techniques for the measurement of the activity of the enzyme. A reappraisal of the distribution of polyphenol oxidase in higher plants would be most timely.

For several plant tissues, such as tea leaves (Sreerangachar, 42), potato tubers (Boswell and Whiting, 11; Baker and Nelson, 8), and spinach leaves (Bonner and Wildman, 10), polyphenol oxidase has been designated as the principal terminal oxidase in respiration. Recently expressed doubts (Schade et al., 39; Levy and Schade, 28) as to the position of this enzyme in the respiration of the potato do not seem to be fully justified. The criticism of Schade et al. (39) regarding the use of catechol has been dealt with previously. One of the principal arguments of Levy and Schade (28) against the participation of polyphenol oxidase in the respiration of potato is the evidence from their series of experiments on carbon monoxide inhibition. In a copper system the carbon monoxide inhibition should not be light-reversible, yet these authors' data indicate that carbon monoxide inhibition is reversed by light. But the results were not entirely consistent. In six out of twelve experiments irradiation appeared to have irreversibly counteracted carbon monoxide inhibition, for turning off the light did not restore the inhibition. In other experiments, preillumination seems to have protected the potato slices against subsequent carbon monoxide inhibition. It would appear that light altered the physiological status of the material, and the interpretation of the carbon monoxide inhibition data becomes uncertain.

Levy and Schade (28) have also presented evidence for the existence of two other oxidases in the potato. The addition of beef cytochrome C to potato homogenates caused an increased oxidation of p-phenylenediamine and of ascorbate, relations suggesting the presence of cytochrome oxidase. A second enzyme present in potato homogenates was able to oxidize p-phenylenediamine and ascorbate directly; this enzyme was not sensitive to carbon monoxide, and its action was markedly reduced by low partial pressures of oxygen. It may be noted in passing that some of the properties of this second enzyme are strikingly similar to laccase, previously discussed (see Keilin and Mann, 22). The presence of other enzymes in the potato is in no conflict with the occurrence there of polyphenol oxidase, a fact which has been established by evidence of the highest validity, namely, the isolation and purification of the enzyme itself from potato tubers (Kubowitz, 24-26).

Bonner and Wildman (10) concluded that polyphenol oxidase is the terminal oxidase in the respiration of spinach. They found no evidence for the existence of either ascorbic acid oxidase or cytochrome oxidase. These conclusions have been challenged in some quarters. Stenlid (43), although conceding that polyphenol oxidase may be the terminal oxidase in spinach, has questioned the evidence on which Bonner and Wildman based their conclusion. The latter workers laid great stress on the inhibition of respiration by p-nitrophenol, which proved to be a stronger inhibitor than o-nitrophenol. According to Stenlid the reverse result would have been of greater significance, since orthophenols are natural substrates for the enzyme. Stenlid has also found that p-nitrophenol is a general inhibitor of respiratory enzymes. It inhibits oxygen consumption in carrot tissue, in which presumably a cytochrome system is operative. Unlike

Bonner and Wildman (10), Rosenberg and Ducet (38) found that spinach leaves contain a cytochrome oxidase as well as a polyphenol oxidase. The addition of horse heart cytochrome C quadrupled the rate of oxidation of p-phenylenediamine by spinach leaf homogenates. In a parallel trial a vigorous oxygen uptake was also obtained upon the addition of catechol.

In attempting to evaluate the physiological significance of polyphenol oxidase in respiration, one is confronted by the presence in the cell of alternate pathways for terminal oxidation. The question of greatest physiological interest is, of course, which terminal oxidase among the several present, is responsible for most if not all of the oxygen absorption during normal respiration. For many plant tissues the answer to this question must await further experimentation.

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DISCUSSION

DR. NASON: One approach to the problem, as I see it, would be to attempt to induce individual metal deficiencies, including copper, and then analyze your particular system and possibly others in the deficient plant. If the activities of these enzymes were affected by a copper deficiency and then restored to normal by the addition of copper, this would give a lead to one of the functional significances of this element.

DR. ARNON: That, of course, would be a very neat demonstration. I should add that our particular program is to undertake to analyze the functions in the normal plant. Our next step in the program is to see, of course, what happens when the system is deranged. It might perhaps be simpler to try to learn something about the normal system. It may prove that the other way will be a short cut.

Dr. Steward: There was one idea that I intended to inject earlier in the discussions. This is related to the possible function of copper in oxidase activity through the quinone system. We do not have to be tied to the role of the system in carbohydrate breakdown. Copper may have other equally important roles in the metabolism of nitrogen through the ability of the orthoquinones to deaminate amino acids. Secondly, I would like to ask whether the Hill reaction has been shown to be enzymatic. We must recall that the first discovered effects which led up to the Hill reaction were obtained under conditions such that enzymes were precluded.

Dr. Arnon: I will attempt to discuss your first point first. I fully agree that there is no reason to limit copper catalysis to any one process in the plant. Reference was made here to the copper-containing indoleacetic acid oxidase which has been isolated from roots. An attempt to link this particular enzyme with the metabolism of the green leaf is no attempt to place an exclusive function for it in the leaf, where some important processes go on which we know very little about. Some, of course, are catalyzed by the enzymes involved in photosynthesis, and perhaps copper may be involved here too. Yet copper is by no means confined to the green leaf. As far as the Hill reaction is concerned, I don't think there is the slightest doubt about the enzymatic nature of this reaction. The system is extremely sensitive to heat. Heating it to 40 degrees for 2 or 3 minutes will completely knock it out. Studies with specific inhibitors also confirm the enzymatic nature of the Hill reaction. The earlier observations that oxygen evolution was obtained from dried heat-treated material when exposed to light have now been explained on the basis that residual organic peroxides were present. The great contribution of Hill is that he demonstrated that oxygen evolution depended upon a hydrogen acceptor. Without added hydrogen acceptor, the reaction does not proceed appreciably. Isolated chloroplasts exposed to light may give off 1/10 or 1/100 of mm^3 of oxygen, but when illuminated under proper conditions with a hydrogen acceptor there is a great quantitative evolution of oxygen.

Dr. Steinberg: Would you care to make a statement on the present methods of purifying solutions, that is, for removing trace elements, and as to whether there is any evidence of other essential elements to be added to our list?

Dr. Arnon: As far as purification methods go, for copper, zinc, and manganese we still use calcium carbonate precipitation. You have already mentioned the 8-hydroxyquinoline method. We find that for molybdenum we get good purification by the use of copper sulfide. The copper sulfide seems to carry the molybdenum with it. The copper sulfide is formed by passing H_2S through the medium containing copper nitrate. In this case, we have to get rid of the copper sulfide and the excess H_2S. Obviously, this method would be unsuited for copper studies. As to whether an element is essential or not, we have taken the point of view which does not permit us to answer this question in a yes or no manner. We would like to look on this as a quantitative, rather than a qualitative question. For example, suppose that we were to ask whether cobalt is essential for plants. I believe that at present a categorical answer to that would be unjustified. All that we could say is as follows: We are aware of the limitation, namely that we can never remove the last trace of cobalt. If the requirement for cobalt by a given plant is sufficiently small so that its needs can be met by the amount of impurity still residing either in the nutrient medium or in the seed or in both, then it may be experimentally impossible to prove by our present techniques the necessity for cobalt. However, if in the future we are able to improve our techniques or, on the other hand, find a plant whose requirements for cobalt are high, then we may be able to answer the question in a yes or no manner with regard to the essentiality of cobalt in the nutrition of plants. If this view is carried to its logical conclusion, we would say that it is impossible to prove that any element is not essential. It is, of course, possible to prove that an element is essential. Any element which is commonly found in the plant should be suspected of being essential.

Dr. STEINBERG: In other words, you do not believe in negative results?

Dr. ARNON: That's right. I believe that negative results in this field are not convincing, because it is impossible to rule out chance contamination and a host of other factors. I will say this: I do not wish to give the impression that I would accept one sporadic positive result. We would insist on consistency in positive results, but consistency in negative results would not be suitable.

Dr. BALLENTINE: It might be pointed out that Dr. Arnon's second approach, namely the isolation and characterization of systems in which the element is functioning, would necessarily bypass the necessity of demonstrating the essentiality of a given element in the growth of the organism.

Dr. ARNON: I think you are quite right. That is a very good point. For example, if we could show that higher plants need vitamin B_{12} we would then have a function for cobalt without having to go through the laborious procedure of removing cobalt from the nutrient medium. However, we have not as yet discovered an essential function of an element via the biochemical route. At the present time, although we know that many elements are essential for the growth of plants, we are not aware of the systems in which they function.

Dr. NASON: With respect to the possible function of polyphenol oxidase as a terminal oxidase in higher plant respiration, it seems to me an important point that neither carbon monoxide nor cyanide knocks out completely aerobic respiration of higher plants. This is one of the arguments that has been used against the cytochrome system itself. How do you reconcile the experimental fact with these postulations?

Dr. ARNON: We might say that the same applies to cytochrome; yet I think most biochemists would not challenge the role of the cytochromes in cellular respiration. The evidence for polyphenol oxidase, so far as cyanide inhibition is concerned, is no better and no worse than that for the cytochrome system. We have to explain not only the failure of cyanide to knock out polyphenol oxidase but also the failure of cyanide to knock out the cytochrome system if it is the sole terminal oxidase. In other words, additional experiments are necessary to explain this differential inhibition. There has been some speculation on this point. One explanation is as follows: that the cell has several alternative pathways. When one pathway is inhibited, the electron transfer is shuttled through an uninhibited pathway. It has been suggested that possibly the flavine systems may function as one of the nonmetal bypasses. Another hypothesis which has been advanced to explain this differential inhibition is that of the relative concentration of the enzyme in the cell. The idea is that you can never poison all of the enzyme. Even though you use a high concentration of cyanide, a fraction of some of the metal oxidases may still be functioning.

Dr. BOWEN: Isn't it possible with respect to your third criterion for essentiality that the plant cannot absorb a sufficient amount of the micronutrients from the soil even if it is present? Have you ever found that the plants could obtain micronutrients through the leaves?

Dr. ARNON: Yes, they seem to be able to do so, even for such elements as nitrogen. They use, for example, urea sprays commonly now in agriculture to apply nitrogen through the leaves. As far as micronutrient nutrition in the field is concerned, out west this unorthodox method of application is the one used commercially, because our soil has such high fixing power for some of these micronutrients, and one would have to use uneconomical amounts if you applied the micronutrients directly to the soil. For example, in the peach orchards enough zinc has been applied to the soil to theoretically last the plants for 40,000 years; yet the trees still show acute zinc deficiencies. We would have to apply a concentration in the soil which would theoretically be sufficient for 200,000 years before the plants had sufficient zinc. By applying a very small amount, however, through the leaf the deficiency can be eliminated. There are techniques used in agriculture such as driving zinc nails into trees. In fact, guns have even been devised for driving zinc slugs into trees. All of these methods have been effective. There is the problem of competition between the soil and the plant, and one must often bypass the soil in applying the micronutrients.

Dr. Baer: I take it that you have no objection to applying the trace element through the root so long as the roots are not in the soil?

Dr. Arnon: I am afraid that that is a loaded question!

Dr. Steinberg: Do you get any injury with iron sprays?

Dr. Arnon: It is a question of concentration. I might say that it doesn't always work. To my knowledge there has been no good cure for iron chlorosis for pear trees in California by the spraying of iron onto the leaves. Actually this condition is corrected by placing the dry iron salt into a small hole bored into the trunk of the tree. They have used iron spray in Hawaii for the pineapple iron chlorosis and have obtained good results.

Dr. Kaplan: Is there any evidence that you obtain a stimulation of oxygen uptake in plant homogenates when you add catalytic amounts of quinone?

Dr. Arnon: Yes. Bonner, using spinach leaves, observed the stimulation of oxygen uptake when a small amount of quinone was added to homogenates.

Dr. Kaplan: Does it also stimulate CO_2 production?

Dr. Arnon: Yes, CO_2 production was also stimulated. However, where he added catechol, the CO_2 evolution was knocked out, indicating some sort of injury.

THE COPPER NUTRITION OF GREEN PLANTS AND FUNGI

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The Era of Chemical Stimulation

The necessity for copper in the nutrition of plants did not emerge as an isolated and clear-cut discovery. The chemical elements essential for green plants originally included carbon, hydrogen, oxygen, nitrogen, potassium, phosphorus, magnesium, sulfur, calcium, and iron. Pasteur in his fermentation studies had determined that yeast could grow on a culture medium containing sugar, an ammonium salt, and yeast ash. Raulin was assigned the problem of identifying the biologically essential chemical elements in the yeast ash. Nutrition studies with another fungus, *Aspergillus niger* van Tiegh., were undertaken for this purpose. The reasons for the selection of *A. niger* are uncertain, but Pasteur must often have seen it growing in his father's tanning pits. Raulin succeeded without benefit of aseptic methods in demonstrating that *A. niger* requires almost exactly the same elements needed by green plants. While calcium did not appear to be necessary for growth of the fungus, zinc and probably manganese and silicon did appear to be required.

These experiments of Raulin led to a controversy that lasted a quarter of a century. Only the French investigators, particularly Bertrand and Javillier (9), accepted Raulin's interpretation of his results. Pfeffer, and most investigators, considered the increased yields obtained with zinc and manganese to be due to chemical stimulation or irritation. Many papers soon appeared presenting data that apparently demonstrated a similar action by traces of a wide variety of poisonous substances, including copper. Introduction of the calcium carbonate method of purification of nutrient solutions by Steinberg (68) in 1919, however, conclusively demonstrated that the theory of chemical stimulation has no basis in fact, for the traces of iron and zinc added to the nutrient solution were found to be indispensable for growth and sporulation of the fungus. Traces of other chemical elements, including copper, manganese, molybdenum, boron, and probably gallium and scandium, have also been considered indispensable for the growth of plants.

The results obtained with chemical elements in nutritional investigations have been summarized, in an attempt to correlate biological essentiality and atomic structure by Steinberg (72). Table 1 is a subtable based on the location of each biologically essential element in a chemical periodic table arranged exclusively on the basis of shell, subshell, and atomic number. It will be noted that biologically indispensable elements required in large quantities, i. e., macronutrients, have lower subshell numbers and intermediate atomic numbers. The nutritive essential elements needed only in traces have higher subshell numbers and more extreme atomic numbers. The segregation of these elements into the triads of subshell 1 = 0; into the diads of subshell 1 = 1; and into the monads of subshell 1 = 2 are additional confirmation of a basic relation between biological essentiality and atomic structure. The mathematical probability that a random selection of twenty-two elements on the basis

TABLE 1

RELATION OF BIOLOGICAL ESSENTIALITY OF THE CHEMICAL ELEMENTS TO ATOMIC STRUCTURE

Biologically essential	Sub-shell number	Rank (ascending atomic numbers)*									
		1	2	3	4	5	6	7	8	9	10
Macro-nutrients	1-0	H, Na, K	Mg, Ca,—								
Intermediate	1-1	B, Ga	C, Si	N, P	O, S	Cl, I					
Micro-nutrients	1-2	(Sc)	*	*	Mo	Mn	Fe	Co	*	Cu	Zn
(Rare earths)	1-3	. .	. .	. .	. .	. .	. .	. .	. .	. .	. .
Total number per rank	1-0-3	6	4	2	3	3	1	1	0	1	1

* Rank 2: Ti, Zr, Hf, or Th; rank 3: V, Cb, Ta, or Pa; rank 8: Ni, Pd, or Pt.

of biological indispensability, in a total of ninety-two chemical elements, would fall into an ordered and coherent arrangement is probably infinitesimal. Not more than three or possibly four more of the chemical elements are likely to be found biologically essential in the future, according to this table of correlations.

EXPERIMENTAL METHODS FOR INDUCING COPPER DEFICIENCY

Steinberg (68-70) showed that trace element impurities (" heavy metals ") could best be removed effectively by adding calcium carbonate to the nutrient medium, heating, and then filtering. Precipitation of impurities as phosphates and carbonates and their adsorption on the precipitate of dicalcium phosphate results apparently in an almost complete removal of trace element impurities. A single treatment is effective with relatively pure nutrient components and water redistilled in a pyrex glass still. Hughes (31) considered this purification process highly satisfactory for the preparation of pure standards in spectroscopic analysis. Pyrex ware was found to be suitable for trace element studies by Steinberg (68).

Bortels (10) introduced the use of adsorbent charcoal for the removal of trace elements and considered it to be as effective as calcium carbonate. Both methods were effectively employed by Roberg (54, 55). In a comparison of the two methods by Steinberg (70), the carbon method appeared to be somewhat more effective for the removal of traces of copper impurities, but objectional because of its introduction of other impurities.

Various other modifications of the purification procedure as applied to the nutrient solution as a whole have been proposed. Hopkins (30), working with *Lemna minor*, found that precipitation of dicalcium phosphate in the nutrient solution was unnecessary for removal of manganese, but that the previously prepared reagent could be used. Sakamura (58) used a somewhat similar technique based on the use of thoroughly washed calcium phosphate. In this procedure, the process of washing probably results in formation of the dicalcium phosphate. Shu and Johnson (62) have recommended removing the impurities from glucose by precipitation of aluminum hydroxide with ammonia in the solution and then filtration.

The calcium carbonate procedure has also been adapted for the removal of trace elements from nutrient solutions for higher plants by Stout and Arnon (77). The individual salts are treated separately in the presence of phosphate ion and the dithizone test used to check on the absence of trace element impurities. Piper (52) took the additional precaution of extracting with dithizone after calcium carbonate purification and then crystallizing the individual salts. Extraction of copper with dithizone has been reported to be satisfactory by Hoffman (29).

Another chemical procedure has begun to be used in the study of trace element requirements, namely, the addition of organic componds to the nutrient medium for the purpose of forming unionized heavy metal complexes or chelates. This possibility was called to the attention of investigators by Zentmeyer (85), who pointed out that 8-hydroxyquinoline (oxine) exhibits fungicidal action because of this chemical property. Effective use of oxine has also been made by Hewitt, Jones, and Nicholas (26) for removing molybdenum from nutrient solutions for higher plants by the precipitation of an iron-molybdenum complex. The residual traces of oxine are claimed to be without effect on the plant. According to Lundell and Hoffman (39), the same procedure should also remove completely a total of twenty-three chemical elements, including iron, zinc, copper, gallium, and molybdenum. Another fifteen elements are partially precipitated, including manganese and scandium.

Minimal yields relative to controls obtained in mineral deficiency studies in aseptic culture are listed in Table 2. Results with four forms—a fungus and two species of higher green plants—are tabulated. Excellent results with macronutrients, i. e., biologically essential chemical elements required in greater quantity, are readily attained without special purification processes. The results for micronutrients, i. e., biologically essential trace elements, were obtained with calcium carbonate purification. Examination of the data on micronutrients will disclose that results with copper are not as good as those with most of the others. In order to obtain even these results, however, it was necessary to subject the sucrose to special preliminary treatment with adsorbent or powdered magnesium before nutrient solution purification. An interesting phenomenon probably based on the retentiveness of sucrose for trace elements is that the purification process is capable of giving improved results when all inorganic components of the medium are spectroscopically pure. Evidence is available that for best results a single treatment is not as effective as repeated treatments.

TABLE 2

Minimum Yields Relative to Controls in Mineral Deficiency Studies in Aseptic Culture With Purified Nutrient Solutions

Element omitted	Percentage of maximum yield				Element omitted	Percentage of maximum yield			
	A. niger	*Lemna minor*	Tobacco			*A. niger*	*Lemna minor*	Tobacco	
			Xanthi	Maryland				Xanthi	Maryland
...	100.0	100.0	100.0	100.0	Fe	0	0.7	1.4	0.7
N	0.1	....	....	....	Zn	0	33.3	14.9	34.5
K	5.5*	....	....	....	Cu	23.4	30.2	44.3	73.1
P	0.1	....	....	....	Mn	12.8	1.6	1.6	1.5
Mg	0.5	....	0.4	....	Mo	2.8	4.4	76.2	2.9?
Ca	100.0	0.4	0.8	0.2	Ga	37.7	53.2	72.3	83.5
S	2.4	....	....	....	B	100.0	8.9	3.1	9.6
					Sc	37.5	100.0	100.0	100.0

* In presence of sodium ion.

This description of methods has made no reference to recrystallization, biological purification, and electrolytic removal of impurities. The reason is that none of these have been of great aid in determining trace element requirements. The work of Wolff and Emmerie (82) might be discussed as an illustration of this reaction. Electrolysis of the nutrient solution of *A. niger* causes a loss of spore color that can be remedied by the addition of a copper salt. No diminution or increase in yield took place. Biological purification led to similar results, except for a very slight increase in yield in the presence of copper. Despite the supporting evidence obtained by the estimation of copper with urobilin, these results disagree with the work of other investigators. Reproduction, in the event of a deficiency of any essential element, is never interfered with prior to a more or less marked inhibition in growth.

The difficulties in approaching a complete removal of trace elements are very great. As an illustration, the purest sample of iron that could be produced by the Westinghouse Electric and Manufacturing Company (1) was 99.99 per cent pure and still contained spectroscopic traces of 27 elements. Included were zinc, copper, manganese, molybdenum, gallium, boron, and cobalt. The contaminations in chemicals, added to those leached from the containers plus those introduced with spores or seeds, would indicate that complete removal of a micronutrient has never been attained.

The Copper Nutrition of the Fungi

Among those who have succeeded in demonstrating increased yields under the influence of minute quantities of copper was Gunter (23). This investigator worked with *Mucor corymbifer, Rhizopus nigricans,* and *Botrytis cinerea.* Similar results were obtained by Ono (51) and by Kanter (33) with *Aspergillus niger.* The differences in yield were slight and were attributed by these investigators wholly to chemical stimulation.

Bortels (10) appears to have been the first to demonstrate that copper exercises a nutritive role in an organism. *A. niger* could be made to suffer a 50 per cent loss in yield on omission of copper, and its spores remained brown. Addition of copper gave normal yields and black spores. These results were verified by many investigators, beginning with Roberg (54, 55). Other fungi behave similarly. Almost identical responses were obtained by Metz (43), using ten species of fungi. Losses in yield on omission of copper were obtained, along with a loss in color of the spores of *A. niger* and the mycelium of *Fusarium.* Steinberg (69) found copper to be essential for the growth and sporulation of *A. niger* in the presence of added manganese. Mulder (47) reported that responses to copper occur in *A. glaucus, A. flavus,* and *Penicillium glaucum* in respect to both growth and spore formation.

McHargue and Calfee (40) noted that the addition of copper to the medium slightly decreased total nitrogen and protein nitrogen in *A. flavus* as compared to deficient cultures, whereas the fat content was increased. Manganese had a similar effect. The same investigators (41) also determined that an addition of either copper, manganese, or zinc to the medium causes increased growth and respiration in aerobic yeast cultures. The cells are smaller in the presence of copper. Though no data were presented for the effect of copper alone in anaerobic culture, its addition together with manganese and zinc caused a ten-fold increase in the formation of carbon dioxide. It might therefore be concluded that the effect of these elements was greater on carbon dioxide production in the absence of oxygen.

Some interesting results on the metabolism of copper in *A. niger* have been reported by Gollmick (21). The brown spores formed in copper-deficient cultures became black as usual upon subsequent addition of this element. If, however, the air in the culture flask was replaced by hydrogen or nitrogen prior to the addition of copper, the spores remained yellow. Atmospheric oxygen is apparently required for the formation of aspergillin in the spores. The previous results of Schwartz and Steinhart (61) had demonstrated the absence of copper in this pigment. Among other data given by these two workers are the facts that the copper in the cells of the fungus is partly leachable with water; that copper absorption increases with acidity; and that the nitrogen balance in plus and minus copper cultures is the same. Autolysis of plus copper cultures led to the presence of high ammonia and low amino contents in the culture solution, as compared to minus cultures. An article by Steinberg (73) has given data revealing that the effects of copper and other micronutrient deficiencies of cultures are increased when these are grown in carbon dioxide-free air.

Copper has also been reported to be essential for *Ceratostomella ulmi* by Ledeboer (35), for *Trichophyton interdigitale* by Moser, Saunders, and Kingery (46), and for *Phymatotrichum omnivorum* by Rogers (56). Yoshimura (84) studied the copper requirements of eight species of *Aspergilli* and decided that this element participates in the formation of spore pigments. Unpublished data of the writer upon a group of six fungi pathogenic to the tobacco plant indicate that copper is required by all. Decreases in yield with copper deficiency varied in experiments from 10 to 30 per cent. The fungi included *Fusarium oxysporum, Rhizoctonia solani, Sclerotium rolfsii, Pythium irregulari, Cercospora nicotianae,* and *Thielaviopsis basicola.*

Induced Copper Deficiencies in Green Plants

The course of events in copper studies with green plants has paralleled that already described for the fungi. At first all increases in yield with copper were ascribed to chemical stimulation (Kanda, 32). Many references can be found in the literature to the stimulative action of Bordeaux fungicide, a lime-copper sulfate spray. The stimulative responses to copper on muck soils of onions was described by Felix (17), and those to copper and other trace elements of many crops growing on peat soils in Florida by Allison, Bryan, and Hunter (2).

It was not until 1931, or four years after Bortel's paper on the essentiality of copper, that two papers appeared almost simultaneously in which similar claims were made for green plants. Sommer (66) grew the tomato plant, sunflower, and flax in water culture and reported decreases in yield, upon omission of copper, that were greater than 90 per cent. Lipman and MacKinney (37) found barley unable to seed in water culture without the addition of 1/16 to 1/8 p.p.m. of copper. These claims were soon verified in subsequent investigations. McMurtrey and Robinson (42) obtained copper-deficiency symptoms in tobacco, characterized by a leaf spot and wilt. Brandenburg (11) found copper essential for the nutrition of the oat plant and stated that the deficiency symptoms were identical with those associated with "reclamation" disease. Haas and Quale (24) succeeded in producing symptoms of copper deficiency with Valencia orange trees in sand culture, the symptoms being identical with those of exanthema in citrus orchards. According to Van Schreven (79), beets and oats require copper for normal growth. Tomatoes in water culture required 0.02 p.p.m. of copper in experiments by Arnon and Stout (7). The symptoms of deficiency in plants grown on solutions purified with calcium carbonate included stunted top and root growth, dark bluish-green foliage, leaf curl, and failure to flower.

Deficiency symptoms following omission of copper from nutrient solution or sand cultures have also been reported by Hoffman (29) for oats, Hoagland (28) for plums, and Piper (52) for oats, wheat, *Lolium subulatum, Phalaris tuberosa,* flax, tomato, subterranean clover, lucerne, and rye-grass. Still other reports of successful demonstrations of the essentiality of copper in plant nutrition include those of Bailey and McHargue (8) for the tomato, Wade (80) for the Valencia orange, Millikan (44) for wheat and flax, and Steinberg (74) for *Lemna minor.*

These results render it clear that copper in minute traces is biologically essential for the growth and reproduction of green plants. The not unusual coincidence that the deficiency symptoms determined in solution and sand culture are identical with those of two well-known plant diseases brings additional confirmation for an interpretation of the data as those of a nutritional lack. Toxicity and nutritive capacity are not mutually exclusive properties of the chemical elements.

Naturally Occurring Copper Deficiencies in Green Plants

Practical applications of copper in crop production have been current for many years. The effects of its application, however, have been attributed to chemical stimulation or to protection of the plant against microorganisms. Applications of copper and other trace elements were found by Allison, Bryan, and Hunter (2) to permit the use of peat soils in Florida from crop production. Grossenbacker (22) and Floyd (18) used Bordeaux sprays and copper sulfate to cure diseases of fruit trees that were apparently due to microorganisms. Felix (17) obtained commercial crops of onions on muck soils in New York by heavy applications of copper sulfate. It was only subsequent to the appearance of the publications of Sommer (66) and Lipman and MacKinney (37) that a nutritional interpretation was applied.

Although the use of copper for crop production is primarily for fertilization, the current practices are most conveniently discussed as different phases, omitting its microbial action. Some of the data place emphasis on fertilization, whereas others place it on prophylaxis. Fertilization may be concerned with manifestly abnormal plants, or with apparently normal plants. Prophylactic aspects have been emphasized by investigators in work on reclamation disease and exanthema or dieback of fruit trees.

TABLE 3

THE EFFECT OF COPPER FERTILIZATION ON THE COPPER CONTENT OF VARIOUS PLANTS DRY-WEIGHT BASIS

(From Lucas, Robert E., *Soil Sci.*, 65: 461-469. 1948.)

Crop	Degree of response	Copper in plants, p.p.m.	
		−Cu	+Cu*
Alfalfa†	Fair	5	10
Barley	Good	10	14
Carrot roots	Good	3	5
Dill seed†	Good	6	12
Head lettuce†	Good	3	9
Ladino clover†	None	7	14
Oats (preheading)	Good	11	15
Onion bulbs†	Fair	2	5
Peppermint†	None	8	12
Red clover†	None	7	15
Sudan grass	Good	5	10
Sugar beets (tops)	Good	6	7
Spinach	Good	8	12
Tomato fruit	Fair	4	8
Wheat grain†	Good	6	8
Wheat (preheading)†	Good	8	12

* One hundred pounds copper sulfate per acre.

† Crops grown in the field. Data on degree of response by P. M. Harmer.

The use of copper for remedying deficiencies in organic soils has already been mentioned. A study by Lucas (38) with many crop plants grown on muck soils supplies representative data. Table 3, which is a reproduction of one of this investigator's tables, reveals only three out of sixteen crops that failed to respond. These were Ladino clover, red clover, and peppermint. All crops, whether they responded by improved growth or not, showed an increased copper content as a result of its application as one hundred pounds per acre of copper sulfate. The increases ranged from 1.0 p.p.m. to 8.0 p.p.m., or as much as 100 per cent.

Table 4 has been recomputed from one published by Lucas (38). It presents the results on protein analysis of plants with and without copper deficiency. An increase in protein of copper-deficient plants was shown in every instance. It averaged about 20.5 per cent for all plants analyzed.

TABLE 4

Protein Contents of Copper-Deficient and Normal Plants
Oven-Dried Samples

(From Lucas, Robert E., *Soil Sci.*, 65: 461-469. 1948.)

Crop	Per cent protein content		
	−Cu	+Cu	Increase
Alfalfa	32.6	30.2	7.9
Barley (preheading)	38.9	27.2	43.0
Oats (preheading)	33.2	29.4	12.9
Wheat	34.2	32.9	4.0
Wheat grain	21.5	19.4	10.8
Sugar beets	24.4	19.9	22.6
Tomato fruit	15.9	13.9	14.4
Carrot roots	19.6	13.2	48.5
Average increase	....	....	20.5

Deficiencies with other types of soils also seem to be widespread (Florida, California, South Africa, Europe, Australia) and quite extensive in acreage. It is not surprising therefore that claims have been made that apparently productive farm soils may be slightly deficient in copper. Publications by Churchman, Russell, and Manns (13) and by Gilbert (19) should be consulted in this connection. There is ample evidence, of course, that induced deficiencies of slight extent are recognizable only as moderate decreases in yields.

That abnormality of crops first noticed in cereals and known as reclamation disease has been reported to occur on peat soils in

Europe. Smith (65), working with oats and peas, recommended applications of copper to the soil. He isolated an organic compound by alcoholic extraction of peat soil, and found it quite effective in high dilution in producing the symptoms of this disease with a normal sandy soil. Copper neutralized the toxic action of "gliedine." Sjollema (63) found a low copper content in wheat, rye, and grasses grown on peat soils. He ascribed the abnormalities in plants and cattle feeding on these plants to copper deficiency. The symptoms of reclamation disease were induced in oats grown in water cultures low in copper by Brandenburg (11).

The results published by Brandenburg have been verified by Arnd and Hoffman (4), Hoffman (29), Piper (52), and others. Symptoms of reclamation disease were produced in oats grown on purified sand containing 5 per cent humic acid by Hoffman (29). Harris (25) recently reported the occurrence of this disease in oats grown on a reasonably fertile loamy fine sand in Florida. The yield of oats without copper was 0.1 bushel per acre; with copper, 8.6 bushels per acre. A photograph by this investigator (Fig. 1) illustrates the relative growth without and with copper. The deficient plants display curled leaves with yellow and withered tips, greatly retarded growth, and no indication of flower formation.

Wood and Womersley (83) determined protein in leaves, stem, inflorescence, straw, and grain of oat plants growing on a copper-deficient soil. Protein was higher in each part of the plant under conditions of copper deficiency than it was in the control. Their results are in agreement, therefore, with those of Lucas (38), which have already been discussed.

Exanthema, or dieback, is a disease of fruit trees that results from copper deficiency. The symptoms include dieback of twigs, S-shaped twigs, multiple buds (Fig. 2), formation of gum pockets, and leaf chloroses. Not all symptoms are shown by each kind of tree. Excellent illustrations of these symptoms are shown in a bulletin by Camp and Fudge (12). This disease had been noted in Florida prior to 1916, when Grossenbacker (22) reported that the condition in citrus trees could be cured by applying copper sulfate to the soil. Floyd (18) found that Bordeaux sprays were also effective in curing

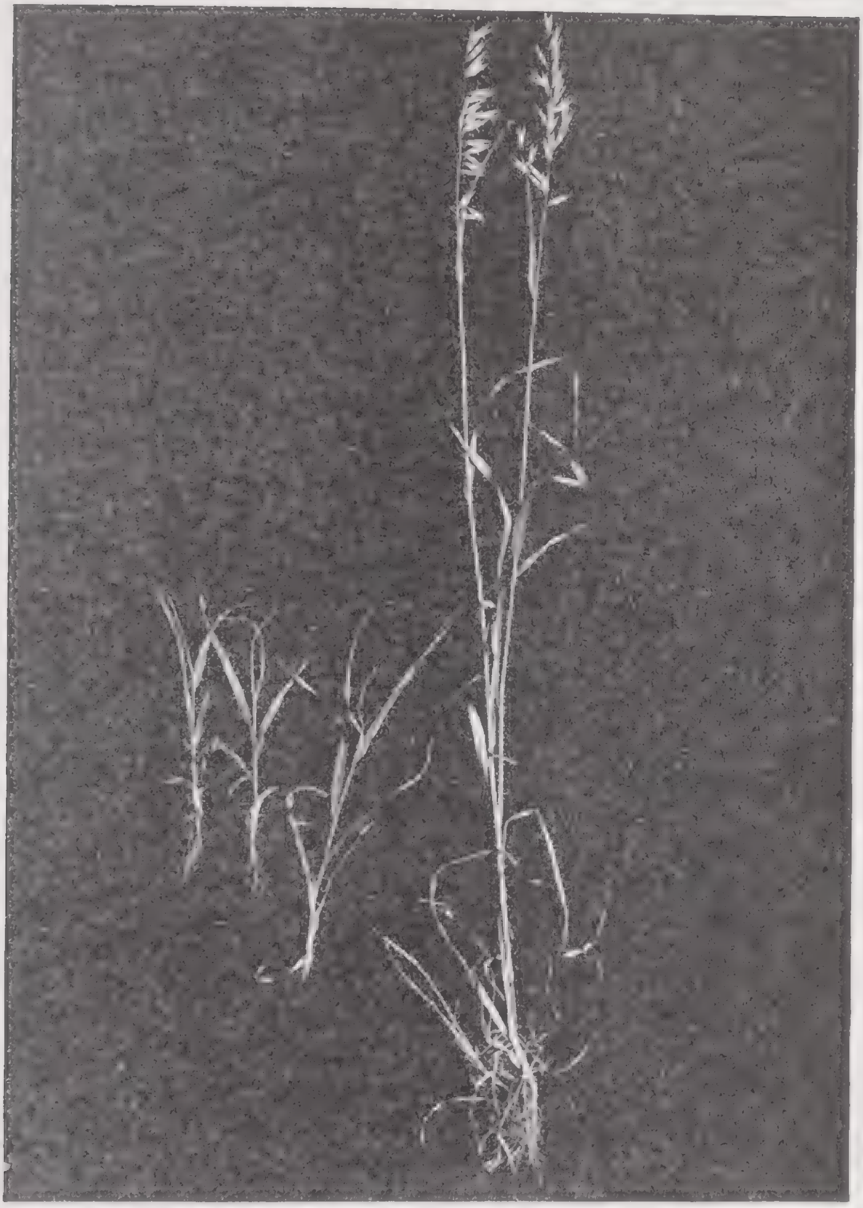

FIG. 1. Oats on May 1, 1947, showing typical plants just before harvesting. Left, without copper; right, with copper. (Courtesy of Dr. H. C. Harris, Florida Agricultural Experiment Station).

exanthema of citrus. Similar disorders of prunes, apples, pears, and olives yielded to treatment with copper, according to Smith and Thomas (64). Anderssen (3) appears to have been the first to realize that the disorder was due to nutritional copper deficiency. He recommended adding 0.25 to 2.0 pounds of copper sulfate to the soil in the immediate vicinity of the tree-trunk. The deciduous

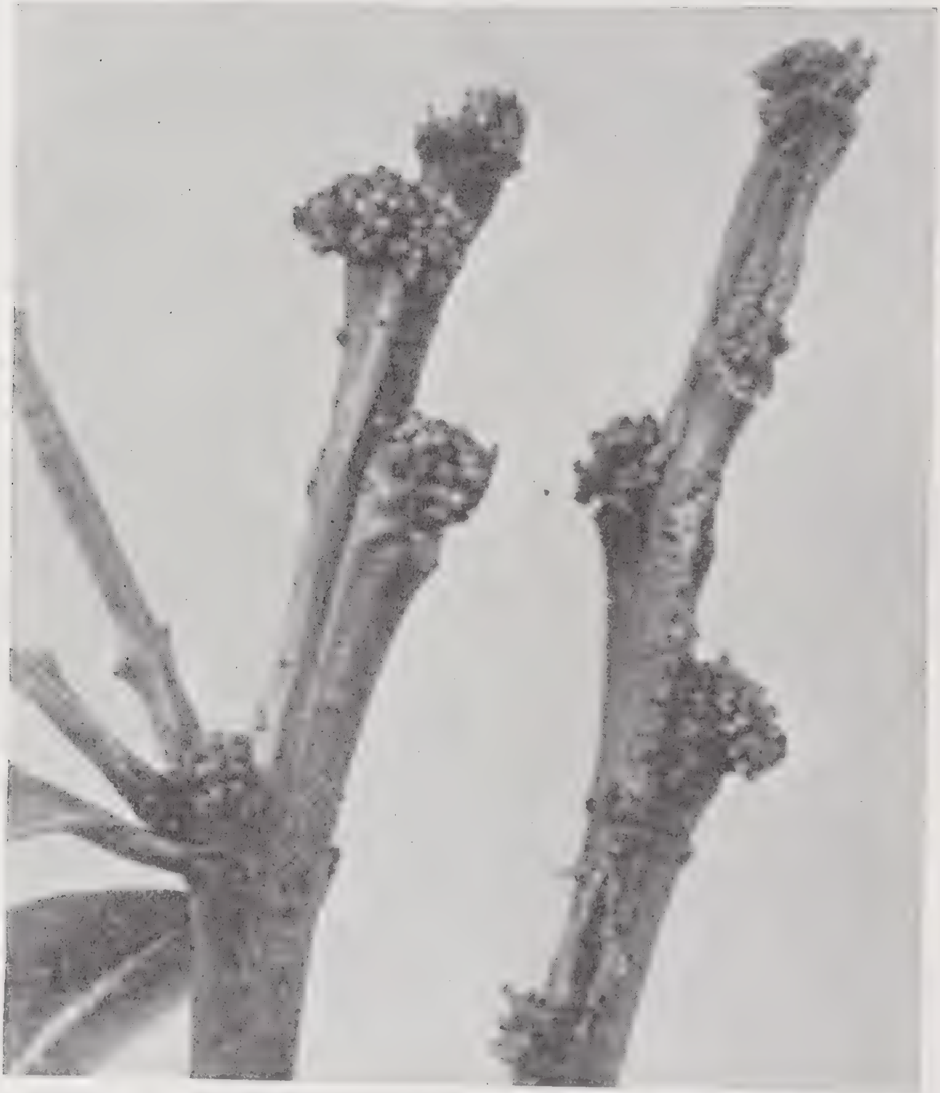

FIG. 2. The multiple-bud phase of dieback on grapefruit shoots showing in addition some bark lesions above at right. (Grossenbacher)

trees with which he worked included pears, peaches, plums, apricots, and apples. Symptoms of deficiency included rosette formation, multiple buds, dieback, and leaf chlorosis. That avocado trees also suffer from dieback has been shown by Ruehle and Lynch (57).

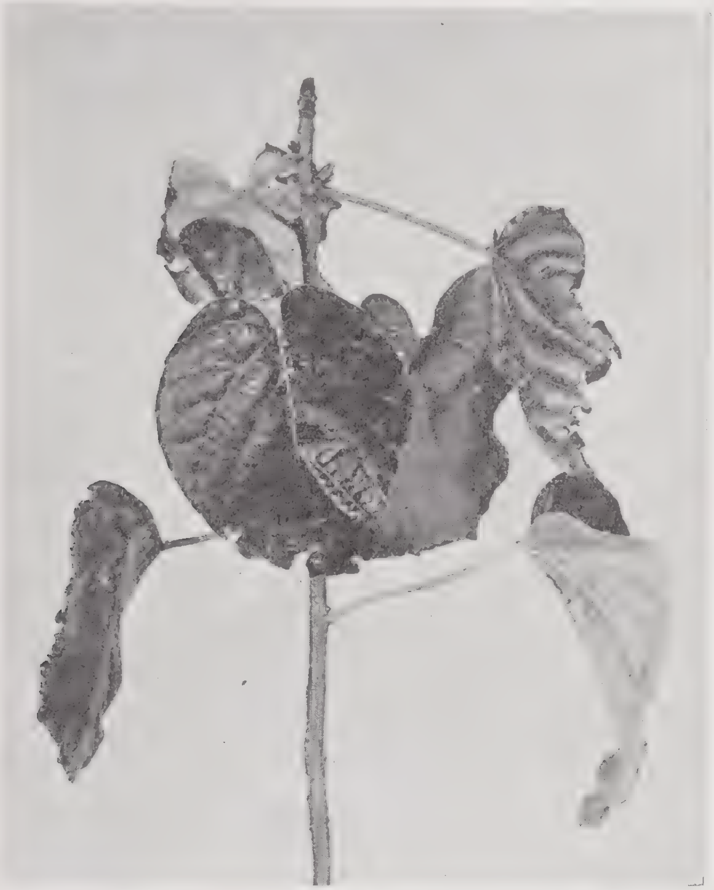

FIG. 3. Shoot of tung tree showing symptoms of copper deficiency—cupping, chlorosis defoliation, ragged leaves, and dead terminal bud. (Courtesy of Dr. R. D. Dickey of the Florida Agricultural Experiment Station).

By non-addition of copper, Haas and Quale (24) produced exanthema in orange trees grown in sand culture. Hoagland (28) did the same with plums in solution culture, and Wade (8) with oranges also grown in solution culture.

The symptoms of copper deficiency in tung trees differ somewhat from those of exanthema. Tung trees with this disorder are described as exhibiting dieback, dwarfing, intervenal chlorosis, and cupping of leaves, marginal and tip burn of terminal leaves, and ragged necrotic leaf margins. This disorder was first described by Drosdoff and Dickey (15). Fig. 3, obtained through the courtesy of Dr. Dickey, illustrates the symptoms in tung trees. Gilbert, Sell, and Drosdoff (20) have made analytical studies on carbohydrate and protein variations of trees suffering from insufficient copper. Some of their data have been reproduced in Table 5 in recomputed form. Dry matter, reducing sugar, starch, and copper were all lower in foliage that displayed mild symptoms of copper deficiency than they were in the controls. Total nitrogen and protein nitrogen were higher than in the controls; and in two out of three instances the copper-deficient leaves were even higher in amino acid nitrogen. Results with the fruits were much the same. Amino nitrogen increased in five out of six trials, and in four instances the relative increases were greater than that in protein.

TABLE 5

RELATIVE CONTENT OF VARIOUS CONSTITUENTS IN THE TUNG TREE IN PARTS DISPLAYING MILD COPPER DEFICIENCY SYMPTOMS

Values given are ratio of deficient/control.
(Computed from data of Gilbert, Sell, and Drosdoff (20).

	Leaves			Fruit		
	June 28	Aug. 15	Oct. 23	June 28	Aug. 15	Oct. 23
Dry matter	0.86	0.93	0.87	0.92	0.90	0.99
Reducing sugar	0.77	0.97	0.77	0.90	1.18	0.65
Non-reducing sugar	0.82	1.02	0.99	1.22	1.26	1.01
Starch	0.64	0.07	0.34	1.07	1.18	1.00
Polysaccharides*	1.19	1.00	1.05	0.74	1.02	0.94
Total nitrogen	1.48	1.33	1.29	1.64	1.64	1.64
Protein-N	1.46	1.32	1.28	1.45	1.51	1.63
Amide-N	1.33	0.33	1.50	2.38	3.67	0.88
Amino-N	1.89	0.43	1.38	2.00	1.94	1.31
Oil	....	....	....	....	0.74	0.46
Copper	0.46	0.55	0.54	0.51	0.64	0.84

* Acid hydrolyzible residue from starch determination.

Dickey, Drosdoff, and Hamilton (14) studied the interrelation between available nitrogen and copper in producing dieback in tung trees. They found that high nitrogen aggravates copper deficiency in tung trees as it has long been known to do in exanthema of citrus.

NUTRIENT SOLUTION BALANCE

A brief reference to interactions between copper and other trace elements, as displayed by plant growth, is appropriate at this point. According to the well known concept of balanced nutrient solutions and ion antagonism, the ratios of ions must fall within definite limits if toxicity to the plant is to be avoided. The ratios would vary widely in comparisons between different ions. Willis and Piland (81) noted that plants growing in soils to which copper had been added might develop symptoms of iron chlorosis. These investigators, however, attributed this response to the diminution by copper of microbial reduction products in the soil and the precipitation of iron in ferric form. While largely due to reactions external to the plant, there was also some evidence of an immobilization of iron in the plant.

Mulder (48) encountered symptoms of "gray speck" disease because of manganese deficiency when excessive quantities of copper were added to solution or sand cultures. An antagonism between copper and aluminum has also been reported by Liebig, Vanselow, and Chapman (36). Odelien (50) has verified the existence of ion antagonism between copper and manganese, and between copper and iron. In studies by Millikan (45) it was found that symptoms closely duplicating those of iron chlorosis are produced in flax by excesses of zinc, copper, manganese, cobalt, or nickel. The addition of molybdenum reduced all symptoms of toxicity caused by these elements excepting dwarfing.

THE FUNCTIONS OF COPPER IN PLANT NUTRITION

The evidence for the metabolic role of copper in plants is quite meager as yet. Increased respiration of yeast, found by McHargue and Calfee (40) to take place upon addition of copper, is to be expected if growth is increased. The increased respiration under anaerobic conditions may indicate, however, that there is a direct relation between copper and oxidation. It is interesting that Arnon (5) also reported a similar relation to exist between copper and aeration in barley. He noted that addition of copper and other micronutrients improved the growth of unaerated cultures of barley with ammonium-nitrogen. The observations of Gollmick (21) that copper is unable to restore the black color to spores of *A. niger* in the absence of atmospheric oxygen may have a similar import.

Assumption of a direct relation of copper to oxidations in the plant is aided by the data on enzymes. Sumner and Somers (78) list the enzymes activated by copper: principally polyphenol oxidase, ascorbic acid oxidase, and cytochrome C.

Emerson and Lewis (16) found that addition of copper and other micronutrients brought about an 85 per cent increase in photosynthesis. The percentage of copper in chloroplasts is higher than in the whole leaf, according to Neish (49), i. e., 74.6 per cent of that in the whole leaf. Chloroplast copper was largely in organic combination. Protein, lipins, iron, phosphorus, ammonium-nitrogen, and catalase were also high in the chloroplasts. Arnon (6) states that chloroplasts of spinach have a high polyphenol oxidase activity. It was reported by Sarata (59) that green tissues in variegated plants are higher in copper than are the chlorotic tissues. Dark flowers of iris and dahlia were also found to contain more copper than the light colored flowers. In another study Sarata (60) reported finding high concentrations in the ovaries and stamens of the flowers. Reed (53) studied the degeneration processes in minus copper leaves of the tomato.

The discovery by Steinberg (75) that individual amino acids produce characteristic abnormalities in plants at high dilutions rendered it clear that symptoms of mineral deficiencies might be caused through the liberation of amino acids into the tissues. Steinberg (76) has pointed out that the evidence would indicate that all the biological elements, with the possible exception of copper, appear to participate in protein synthesis in the plant. The data for micronutrients and protein formation is shown in Fig. 4. Zinc in carbonic anhydrase is implied to participate in removal of carbon dioxide; molybdenum, in nitrate reduction (Steinberg, 71); manganese, in condensation of amino acids to protein (Hewitt, Jones, and Williams, 26); and copper, in protein breakdown. The meaning of the association found by Karp (34) between thiamin and copper content in a variety of plant and animal products has not been made clear.

It is not at all certain that copper does not participate at some stage in the synthesis of amino acids and proteins. It is true that the data here reviewed make it evident that protein increases as a result of copper deficiency. There is some evidence, however, that free amino acids may be increased proportionally in a still greater amount. Additional data are badly needed on both free amino acid and protein content of badly abnormal leaf tissues resulting from copper deficiency. Chromatographic analysis of the spectrum of free amino acid in copper deficiency, and its comparison with those of other micronutrient deficiencies, will probably reveal the existence of chemical differences as characteristic as the morphological differences.

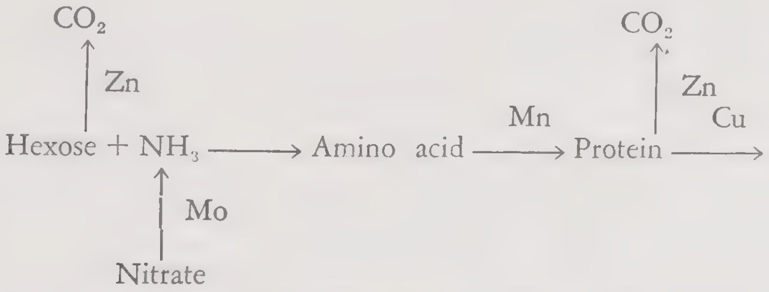

FIG. 4. Enzymatic activations by micronutrients in protein reactions.

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Discussion

Dr. Steward: We have been studying the soluble nitrogen fractions by using the technique of paper chromatography. It is perfectly true to say that the deficiencies of calcium and phosphorus cause changes in concentrations in some of the amino acids, and I would agree with Dr. Steinberg that overall determination of amino nitrogen does not tell us very much. By using the technique of paper chromatography we have been able to find alterations in concentration of hitherto unknown amino acids. For example, some of you may know that recently we have found that γ-amino butyric acid is a very common constituent of plants. The amino group in γ-amino butyric acid is probably in equilibrium with the amino group in glutamine and glutamic acid. This equilibrium is very sensitive to alterations in calcium metabolism. I think it is this type of thing that justifies the remarks that Dr. Steinberg has made. I am sure that it would be a very fertile field to look for the effects of trace elements on specific components of the soluble amino nitrogen. I was interested in Dr. Steinberg's remarks on copper and proteins. I was not sure whether this was an absolute increase in protein or merely due to the fact that some of the nitrogen constituents of the plants were relatively more in the form of protein.

Dr. STEINBERG: If we assume that protein was formed and was used for further purposes, in other words, breakdown, it seems that we must also assume that the absence of copper causes an actual block in this breakdown reaction, so that more protein is accumulated. In the case of the authors that I have consulted there seems to be general agreement that in every case copper deficiency causes an increased protein content to appear in the tissue. The figures I showed are purely relative and depend upon the fraction obtained when one divides the percentage of protein of the deficient plant by that in the normal plant. In all cases this is greater than one in copper deficiency. I take this to mean a real increase in the protein content in the tissue. Other mineral deficiencies have an opposite effect.

Dr. STEWARD: You believe, then, that copper is important in the breakdown of protein?

Dr. STEINBERG: I wouldn't say that I believe it entirely, but I have not been able to find any evidence that it doesn't. The people who have published on this question seem to think it to be the case.

Dr. COMAR: Has there been some question about the accumulation of protein in the roots?

Dr. STEINBERG: In the experiments which I cited in the paper the investigators analyzed all parts of the plants and apparently got an increase in protein in all parts. This is not a problem of translocation, although I will agree that this is a problem which is rather difficult to handle experimentally. I find normally in water cultures that the roots are a very small part of the plant. For example, a tobacco plant weighing from 60 to 140 g. dry weight may have roots weighing about 7 to 8 g. dry weight. An increase of 1% in the whole plant would mean practically no increase in protein quantity in the roots, that is, unless it depended simply on translocation. You might modify those figures, but you could not account for them on this basis.

Dr. GRANICK: I am still not quite clear on the comparison of the copper-deficient plant with the normal. How do you make this comparison? Do you determine the total protein nitrogen of the whole plants? Is it expressed in terms of dry weight?

Dr. STEINBERG: The results are expressed by most investigators on the basis of dry weight, but wet weights have been used in a few instances. The evidence for increased protein is on a relative basis for the analyzed tissue as a whole.

Dr. GRANICK: Of course that does not mean very much, because the plant which has a low cellulose content might have a relatively high nitrogen content on a dry weight basis.

Dr. Steinberg: That is true, and they also differ in their percentage of water. All the authors I have quoted seem to make their own rules for expressing their results, and in reviewing the literature I have found that the simplest method was to divide one by the other and get a relative expression. Some of the authors have used wet material and some have used dry, but in all cases there is a relative increase in protein content. This, of course, is in contrast to all other deficiencies, where there is a decrease in the protein content.

Dr. Granick: I am not, however, entirely convinced that the data signify that there is an increased protein content in case of copper deficiency. It may be that copper deficiency has brought about a decrease in other components in the plant, such as cellulose, which on a dry weight basis, would give a high protein figure. Specifically, do you have data to show that under similar conditions one whole plant low in copper has more total protein than one whole plant high in copper?

Dr. Steinberg: I think that the reported data indicating an increase in total protein for copper are reliable. Of course, there is this point: total protein should be based on the organism or its individual parts as a whole and not on one corrected for cellulose, moisture, or some other single constituent. A better criterion might be one based on protoplasm. I am not saying that the comparison of the total protein in the plants or its parts as a whole is necessarily best, but I am saying that by using this procedure as a basis of comparison, I have obained uniform increases in reported results with free amino acid nitrogen and decreases of protein nitrogen in the case of almost every element, with the exception of copper.

Dr. J. E. Dawson: Dr. Steinberg, if the results had been expressed in terms of per cent of total nitrogen, would this have affected the interpretation of the results?

Dr. Steinberg: I don't know, but see no particular advantage in stating it in that form. Some of the investigators have so expressed their results, but as far as I recall, expressing it in this manner did not lead to an altered interpretation. "Total organic" rather than "total" nitrogen would serve as a better criterion.

Dr. Cunningham: I would like to ask a question concerning the interrelation of copper and molybdenum on pigmentation of *Aspergillus niger* spores. Soil microbiologists determine available soil copper by measuring the depth of pigmentation in *Aspergillus niger* cultures. This method works fairly well, in the hands of my colleagues, for mineral soils, but does not give reliable results for organic soils. There is definite evidence of copper deficiency in such soils and in plants grown on them, yet the *Aspergillus* rating indicates that available copper is high. The molybdenum content of the soils is higher than normal. Could this extra molybdenum exert any effect on pigmentation of the spores?

Dr. STEINBERG: I visited the Florida Everglades Experiment Station recently, and Dr. Allison showed me a large number of kodachromes demonstrating copper deficiency. I was struck by one fact, and that is that the symptoms of copper deficiency of annual dicotyledonous plants consisted only in reduction in yield; no chlorosis or other specific symptoms appeared whatsoever. My interpretation of the reason for that is tied up with the effects of abnormal increases of amino acids. The lack of specific symptoms in the plant is due to the fact that you are working in a highly organic soil. The entrance of much organic material into the plant may interfere with the development of specific symptoms such as chlorosis. I have no direct evidence for this statement. I think if this is true, it would explain why we don't get these symptoms in dicotyledonous plants grown on peat soils. Oats show quite characteristic symptoms both in the peat soils and in the case of inorganic soils, and I do not known why the distinction should exist between the two types of plants. Presumably also the organic copper complexes in peat are much more readily decomposed by *Aspergillus niger* than by the green plant. Other fungi may exist that approximate the green plant more closely in this respect.

Dr. CUNNINGHAM: Does molybdenum affect the pigmentation of *Aspergillus niger?*

Dr. STEINBERG: I have been able to reduce molybdenum to a point where the yield would fall off 93%. The spores that were formed were still black. I take it that this means that molybdenum has no influence on spore color. This black material has been called *aspergillin,* and apparently it does not contain copper. However, copper and oxygen are necessary for its formation.

Dr. THOMPSON: Dr. Steinberg, is there any evidence that copper deficiency in tobacco has any influence on the curing of tobacco?

Dr. STEINBERG: Greenhouse tobacco is of no commercial value. Perhaps Dr. McMurtrey would like to answer that question.

Dr. MCMURTREY: We have always been looking for these specific effects; and in the field it is observed, of course, that where these deficiencies are severe additive effects occur which usually make the crop non-marketable. If there are any elements that you can tie up with quality, potassium and nitrogen seem to dominate the picture in the case of tobacco.

A BROWNING REACTION INVOLVING COPPER-PROTEINS

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In so far as it concerns foods, the Maillard reaction and its effects represent a problem of relatively recent but, nevertheless, intense interest. Much of this interest and the resulting investigative work on the Maillard reaction, which is more generally referred to by the food industry as the browning reaction, was stimulated by the late war, which necessitated the development of combat rations of concentrated nature. These rations had to possess such properties as to retain both nutritional and palatable stability while subjected to extremes of time, temperature, and humidity. Both enzymatic and non-enzymatic browning reared as formidable but little understood barriers.

It is not my intention to discuss the Maillard reaction in general, but rather to discuss a single phase of the browning picture which was brought to light only recently, yet which is profoundly significant. The role played by copper-proteins, and undoubtedly by other metal proteins, in the browning of foods has implications for certain biological reactions. It is because of these implications that I feel that a discussion of copper-proteins in foods is appropriate at a symposium on copper metabolism.

It will be recalled that the first concepts of the browning reaction focussed on amino acid—carbohydrate interaction as the primary functional reaction responsible for the non-enzymatic browning of foods. In a relatively short time the browning reaction has become recognized not as a single group reaction but as a multitudinous complexity of reactions classified into the two categories of enzymatic browning and non-enzymatic browning.

As investigations on the browning of foods increased in complexity, it was not unusual for the catalytic effects of traces of metals to enter the picture. In food chemistry it is well recognized that off-flavors, off-colors, oxidation, and other deteriorations are frequently due to the presence of minute amounts of metals which act as catalysts. Copper, in particular, is an offender in this respect. Its possible relation to the browning reaction was a natural consideration. This problem was attacked on a fundamental basis since the objective of the study was to ascertain, in so far as possible, the mechanisms of whatever reactions were involved.

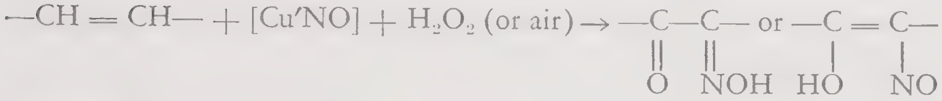
Before going further, it is essential that the line of reasoning followed in the investigation should be made clear. The following discussion will summarize the background information which is prerequisite to an investigation of copper proteins and browning.

In previous work conducted on milk we had found that, under normal conditions of pH, the copper exists in that product as the bound and not as the electrovalent metal. It was also known that as the pH is lowered the concentration of the electrovalent copper increases. In fact, there now exist analytical methods for the determination of copper which depend on the loss of copper by proteins when the pH of the sample is reduced to 3.0 or less (1, 3). It was also ascertained that under normal conditions, i. e., at pH 6.5 to 6. 8, the copper in milk is bound by the protein and that it is undoubtedly distributed quite uniformly with the protein nitrogen (6).

As a result of this earlier investigation, we became interested in the activity of copper with proteins in the near neutral pH range of 6. 5 to 7. 0. For all practical purposes, the selection of this pH range limited the investigation to the effects of bound rather than electrovalent copper.

It has been suggested that copper may combine with certain proteins and react in a somewhat analogous manner to ascorbic acid oxidase in the oxidation of ascorbic acid (4). It was reasoned that trace copper activity may be exerted through reactions of this type. A most logical concept of copper activity in biological reactions has been set forth by the late Dr. Oskar Baudisch, who found a simple reaction, called the B reaction, in which copper is almost unique in behaving as a central atom (2). Only mercurous and silver ions can replace copper in the B reaction, and they are less reactive. The scheme below shows the reaction in simplified form and gives as an example the formation of o-nitrosophenol from benzene and the cuprous salt of nitrosyl.

B REACTION (BAUDISCH)



$$-\mathrm{CH}=\mathrm{CH}- + [\mathrm{Cu'NO}] + \mathrm{H_2O_2}\ \text{(or air)} \rightarrow -\underset{\substack{\| \\ \mathrm{O}}}{\mathrm{C}}-\underset{\substack{\| \\ \mathrm{NOH}}}{\mathrm{C}}- \text{ or } -\underset{\substack{| \\ \mathrm{HO}}}{\mathrm{C}}=\underset{\substack{| \\ \mathrm{NO}}}{\mathrm{C}}-$$

Example:

$$[\mathrm{Cu'NO}] + \text{benzene} \xrightarrow{\mathrm{O_2}} \text{o-nitrosophenol}$$

Cupric ions further react with the o-nitrosophenol to form an inner complex cupric salt which is very stable and deep red in color. The benzene may be replaced by many other aromatic compounds or many other compounds containing an ethylene group in a ring structure. Baudisch, in using the B reaction as a probable explanation of the activity of the polyphenol oxidases, regarded the attached copper as bringing about a molecular rearrangement of the polypeptide and in general activating the protein. In explaining the unique specificity of copper in contrast to the other transition elements in the B reaction, he pointed out the following (2).

(1) For no other metallic ion are the magnetic states of the simple (not complex) ions so conspicuous as for the copper ions (Cu^+ ions are practically diamagnetic, Cu^{++} ions are paramagnetic).

(2) Cuprous ions have a tendency to form an octahedral arrangement in space; cupric ions possess a square or tetrahedral structure (Cu^+ ions link six molecules of pyridine, Cu^{++} ions link four).

(3) The valence change of copper takes place easily in a weakly acid solution with an oxidation potential of low voltage, whereas the oxidation potential for the release of an electron from the cations of other transition elements is far too high to occur in acid solutions.

(4) The formation of complex coordinated ions by the addition of nitrogen compounds lowers the potential of copper ions and makes the valence shift still more easily in acid solution.

Since it had been reasonably well established in the earlier mentioned work on milk that copper was bound in the proteins, an assumption was made that the copper probably acts as a copper-protein complex. Assuming further that the bonding of copper in the protein might be similar to that exhibited by the cuprous nitrosyl in the B reaction, a study was set up to determine the possibility of reactions between copper-proteins and certain compounds containing an ethylene group in a ring structure. For the sake of brevity, the results of this study will be presented in summary form (7).

It was found that when a 1% aqueous solution of casein reacted with excess amounts of any of the materials shown in Table 1 at a pH of 6.5 to 7.0 and at room temperature in the presence of copper oxide, those materials indicated as reactive underwent marked color changes, while those indicated as unreactive demonstrated no changes.

TABLE 1

MATERIALS INVESTIGATED FOR REACTIVITY WITH COPPER-PROTEINS (pH 6.5 to 7.0)

Reactive	Non-Reactive
L-ascorbic acid	oxalic acid
reductic acid	glucose
furfural	lactose
furfuryl alcohol	pectin
catechol	
hydroquinone	
phenol	
pectin, acid hydrolyzed	

These color changes ranged from red-brown or yellow-brown to dark brown at the end of 24 to 48 hours. Treatment of the reacting mixtures with acid and alcohol resulted in precipitation of a protein substance which ranged in color from red-brown, in the case of ascorbic acid, to black, in the case of the aromatic compounds. The dependence of the reaction upon all three of the reacting substances was ascertained by eliminating each ingredient in turn. The precipitated substances all possessed " fragrant " or " fruity " odors and upon analysis demonstrated a further similarity to browning end-products in possessing a rather high degree of fluorescence. It is of extreme significance that glucose, which is known to enter into a browning reaction through an amine-aldehyde interaction, proved to be unreactive. This strongly indicates that the browning reactions observed for copper-casein were dependent upon a reaction between the copper-protein and compounds containing an ethylene group in a ring structure, and further suggests the formation of a complex in which copper possibly acts as the central atom. Another interesting fact was that although the analytical data for any given isolated complex were generally uniform, the copper contents varied from as little as 0.3 mg./g. to as much as 14 mg./g., suggesting that the copper combined in the protein complex is in excess of that required for the reaction. This suggestion has been verified as a result of more recent work, in which we have found that these reactions can be carried out with as little as 2 p. p. m. of copper in the reacting mixture. Under these conditions most of the isolated copper-bearing substances contain copper in the concentration of 150 to 200 p. p. m. Yet, there appears to be no diminution of reaction rates or intensities.

TABLE 2

Proteins and Amino Acids Investigated
(pH 6.5—7.0)

Reactive	Non-Reactive
casein	casein hydrolysate (complete acid)
albumin	tryptophane
gelatin	cystine
bacto-peptone	

Table 2 shows the protein substances which were investigated and again lists them as either reactive or non-reactive. Albumin yielded complex end products similar to casein, but there was one marked difference, namely, that casein was generally more reactive toward all of the compounds, whereas albumin reacted immediately with the aromatic compounds and with extreme slowness with ascorbic acid. Gelatin and peptone reacted similarly to albumin. Since neither the complete acid hydrolysate of casein nor the amino acids in which it was deficient yielded complexing reactions, it seems probable that the copper which was responsible for the reaction was bound, not to free polar groups, but through forces existent in polypeptides.

It is indicated that there may be a parallelism between the reaction of the copper-proteins under discussion and those of the enzymes to which copper is specific. Considering the specificity of such enzymes as ascorbic acid oxidase, it becomes obvious that specific types of linkage are involved. On the other hand, when whole proteins react with copper, the copper distribution in the proteins is quite general. This may account for the generalized reactions of the copper-proteins as contrasted with the highly specific reactions of the copper-bearing enzymes. It does seem reasonable, however, that the different proteins would demonstrate some form of generalized specificity for certain groups of compounds on the basis of protein molecular structure. This probably explains why copper-casein is quite generally reactant to all of the compounds studied, while copper-albumin reacts readily with the aromatic compounds but almost not at all with ascorbic acid, casein being a conjugated and albumin a simple protein.

TABLE 3

Analytical Characteristics

Substance Added to Cu-Casein	Visual Color Change	Fluorescence Value	R
Blank	—	faint trace	2.2
Furfural	++	0.15	1.2
Hydroquinone	+++	0.20	1.3
Reductic Acid	++++	0.70	1.3

Fortunately, during the investigation certain analytical characteristics have thrown additional light on these copper-protein reactions. The copper-casein complexes have been examined for their fluorescence and their polarographic properties. A few of the data are presented in Table 3.

The visual color changes indicated take into consideration both the rate of the color change as well as the final intensity. The fluorescence values were obtained on saline solutions of the isolated complexes by the method of Pearce, Thistle, and Reid (5). The value represents the fluorescence equivalent to quinine sulfate and is given in gammas of quinine sulfate per gram of complex. Both the color change and the fluorescence appear to be qualitative indications of the compound with which the copper-casein reacts. There is a good positive correlation between the two sets of values, a pattern not uncommon to browning reactions.

The column on the extreme right shows the ratios of the step heights of the two polarographic waves common to all of the complexes studied. Two distinct and widely separated polarographic waves were obtained. The first had a half-wave potential of —0.25 volts and the second of —1.2 volts. Since the concentration of a given component at a given half-wave potential is a function of the current, the expression for the ratio of the step heights may be conveniently expressed as

$$R = \frac{I(-0.25\ V)}{I(-1.2\ V)}.$$

The polarographic data were obtained from portions of the saline solutions used for the fluorescence studies.

The polarographic data, in contrast to the fluorescence data, do not indicate that the compound contains an ethylene group but, rather, show the completeness of the reaction of any compound that contains an ethylene group. It appears that when the fluorescence has developed to a maximum for a given reaction, R becomes 1.2 to 1.4. When the reaction is not complete, R is greater than 1.4 and falls between the values of 1.2 and 2.2.

One point must be clarified, particularly in respect to the polarographic data. The copper-casein studied in this instance contained an excessive amount of copper (14 mg. Cu/g. dried Cu-casein). Presumably the excess copper is bound by polar groups, while the copper entering into the reaction is bound by other forces occurring in polypeptides. Since investigating the analytical factors mentioned, we have been able to obtain complexes of much lower copper content. However, we have not investigated the polarographic characteristics of these complexes of lower copper content. One of the more interesting future problems will be to investigate thoroughly the polarographic characteristics of these complexes when they are prepared from copper-proteins of varying concentrations. I predict that a study of this nature will have value, inasmuch as it may be possible to obtain an indication of the amounts of copper bound by polar and non-polar groups existing in a given protein.

Thus far the discussion has related to specific copper-protein reactions, which have been under investigation in vitro. One of the more practical findings is that proteins are "avaricious copper grabbers." A neutral solution of protein, such as casein or albumin, will saturate itself with copper when in contact with an insoluble copper compound, such as the oxide, in a relatively short time. This in itself readily explains why foods, particularly foods high in protein, can so easily pick up copper during or after processing, due to surface contacts. For example, certain surface defects in foods packaged in some flexible packaging material may be attributed to the contact of the food with a packaging material containing copper and other metals. This has been demonstrated in the case of print butter, which will develop surface defects due to contact with metal-contaminated parchment in as short a time as 48 hours. The same butter packaged in parchment of low metal content does not develop surface defects over a period of several days.

We have made several practical tests with these copper complexes which are worth mention. At one time we were asked to demonstrate that a browning reaction can be induced to proceed in whole milk as a result of adding a complex to the milk. We prepared a fresh copper-casein-catechol complex containing 175 p. p. m. of copper. Sufficient of this complex was added to the milk to bring the copper content of the milk up to 0.5 p. p. m. Along with untreated milk, the milk so treated was stored at 40 degrees F. Overnight the treated samples turned a definite chocolate brown color. For some indefinite reason, we continued to hold these samples after the objective, a demonstration of browning, had been accomplished. In the usual short time the untreated milk soured and became putrid, but not the samples that contained the complex. Interested in the possible anti-bacterial effect, we continued to hold these samples. After fifteen months, during which there was ample time and opportunity for contamination, the samples had neither soured nor become putrid.

The question immediately presented itself: was there a possibility that free catechol might be responsible for the anti-bacterial activity? This seemed unlikely, since the prepared complex had been thoroughly washed to remove free catechol or other soluble fractions. No tests applied gave any indication of the presence of free phenolic groups.

In one particular instance we were fortunate in obtaining a whole fluid milk that contained a rather high natural copper content of 0.15 p. p. m. The reaction was induced to proceed in the sample without added copper. Within twenty-four hours its color had changed to a deep brown. This sample was kept for above twelve month without any sign of souring or putrifaction.

It is thus indicated that trace amounts of copper in foods are bound in proteins and can result in a browning reaction, the mechanism of which differs from known types of non-enzymatic browning. Of probably as great significance is the fact that the complex end products resulting from these reactions are in themselves excellent catalysts for the Maillard reaction.

The first positive indication of this further catalytic effect was discovered by Dr. Cronheim while investigating the effects of several copper and iron compounds on a Maillard reaction in liver paste. Among these compounds was a copper-casein-catechol complex which we prepared for him. In terms of its copper concentration alone, this complex was more reactive than any of the other copper or iron salts. For this particular study the extent of the Maillard reaction was based on the evolution of carbon dioxide.

Another example of the catalytic effect of these complex end products may be cited in some tests we have made with powdered milk. Small particles of the dried complex were dispersed in powdered whole milk, and the mixture was kept for several weeks. In a few days a definite growth of a browned area around the individual particles was observed. The growth was not rapid, but neither was it expected to be, since the added complex was in a highly insoluble form and the moisture of the milk powder was kept at less than 4 per cent. Copper oxide dispersed in milk powder did not cause a similar reaction.

There remains the question of the activity of other metals in reactions similar to that involving copper. Cobalt, according to our limited investigations, appears to follow a reaction pattern generally similar to the copper reactions. This may be considered strange, since the B reaction is copper-specific. I discussed this with Dr. Baudisch last fall in Chicago just prior to his ill-fated trip west. A possible explanation, one which he was inclined to accept, is that there might be sufficient copper present in the reagents to make it possible for cobalt to replace the copper. This would not be unusual, since many metals will replace copper in the B reaction, once the orthonitroso compound has been formed. It remains to be seen whether or not cobalt will react if there is no copper present. Perhaps dialysis can be depended upon to remove the copper. This problem remains to be undertaken.

SUMMARY

Certain reactions are described which indicate that trace amounts of copper in foods are bound in proteins and can result in a browning reaction, the mechanism of which differs from known types of enzymatic browning. Certain findings indicate unusual properties of the complexes formed during the copper-protein browning.

Continued investigation of these reactions should throw added light on the nature of such copper reactions and will be valuable whether these reactions result in the deterioration of a food, or in an antibacterial activity, or whether by such studies we arrive at a fuller understanding of the role of copper in nutrition.

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DISCUSSION

DR. C. R. DAWSON: What do you mean by copper-protein? Do you mean a mixture of protein and copper?

DR. THOMPSON: Yes. Protein which has been reacted with copper and then washed under normal conditions to be free of residual copper.

DR. C. R. DAWSON: Did you dialyze it?

DR. THOMPSON: No, we didn't dialyze it. What we were interested in, in contrast to the work that you're doing, was to find out what happens in a particular food when it is contaminated with copper during processing, and that was our best way to get at it.

DR. C. R. DAWSON: I am trying to get straight in my own mind the distinction between a mixture of copper ion and protein and what I call a copper-protein, like hemocyanin, where the copper is firmly bound.

DR. THOMPSON: I'm talking about a much more general protein which appear to combine with the copper and hold it both in polar and nonpolar groupings. We have examined powdered milk to which we added up to 15 p. p. m. of copper and found 95 to 99 per cent of the copper in the proteins.

DR. C. R. DAWSON: You mean that you added that amount of copper to the milk?

DR. THOMPSON: We added it to the milk so that after processing it would contain 15 p. p. m. of copper, and we did not find by simple, ordinary tests that we applied that there was any indication of an appreciable amount of unbound copper present.

Dr. C. R. Dawson: What was the nature of the test? How did you determine the amount of copper?

Dr. Thompson: In the case of Coleman's work he used protein fractionation techniques and then washed them thoroughly.

Dr. C. R. Dawson: By washing you mean dialysis?

Dr. Thompson: No, just straight washing of the precipitated complex. Mr. Coleman, can you offer any suggestions on that? You're much closer to it than I am.

Mr. Coleman: The methods we now use to distinguish between bound and free copper make use of polarographic and spectrophotometric techniques. Another approach which has proven particularly useful in our problem involves a comparison, under controlled conditions, of the rate of destruction of ascorbic acid in the presence of copper and complexing agent, such as protein, with the rate of destruction in the presence of copper alone. Our objective is to screen by means of this simple test system a number of naturally occurring and synthetic copper-complexing substances and select those most inhibiting to copper catalysis for use in more complex systems.

Dr. Thompson: I think what you're getting at, Dr. Dawson, is the fact that there is a possibility that some of this copper remains in the complex as copper oxide or as simple ionic copper. The copper which enters into the particular reaction that I have talked about apparently is a copper that is firmly bound. Whether it is a peptide linkage or not, as Baudisch suggested, I am not prepared to say. I would like to think about it as a possibility. However, the polarographic examinations indicate definite copper complexes, rather than ionic copper.

Dr. C. R. Dawson: The point that sticks in my mind is that if you add cupric ion to egg albumen or gelatin it takes about 3 to 4 days before you can remove the copper by dialysis. I am wondering whether your copper is just cupric ions adsorbed on the surface of a colloidal particle.

Dr. Thompson: I rather doubt that the latter suggestion is correct, since we can get the effect with so little copper present.

Dr. C. R. Dawson: How do you explain again the cobalt browning reaction?

Dr. Thompson: According to Baudisch, there was enough copper in the system to start the B reaction.

Dr. C. R. Dawson: Wouldn't you have to have an amount of copper comparable to the other metal?

Dr. Thompson: Not according to Baudisch. The only thing that we can definitely say, however, is that we have tied copper into the browning system, given at least a partial indication of what the reaction for that is, and shown that once this reaction is completed the end-products of the reaction in themselves are general catalysts for the Maillard reaction.

Dr. MALLETTE: The remark by Mr. Coleman has raised the question about the systems involved in your studies. Are you investigating the effects of protein products on the action of copper on ascorbic acid?

Mr. COLEMAN: Yes. Our research involves a study of the role of traces of copper in the oxidative deteriorations in foods which lead to the development of off-flavors. It has been well demonstrated that among the reactions involved in the production of such flavors in milk is the copper-catalyzed oxidation of ascorbic acid and that hydrogen peroxide is a product of this oxidation. We are interested in inhibitors of such copper-catalyzed oxidations and, at present, utilize the rate of oxidation of ascorbic acid in the presence of copper and such complexing agents as proteins in attempting to predict the probable effectiveness of these substances as inhibitors.

Dr. MALLETTE: Is there enough difference in your system from that of King and some of the other workers to account for the increased effect of copper if it combined with protein?

Mr. COLEMAN: The differences in experimental results obtained may be the result of differences in the protein-copper ratio. None of the proteins or amino acids that we have studied thus far has increased the catalytic effect of copper. Russian workers reported that the copper-histidine complex was more catalytic than ionic copper. We observed, to the contrary, that, under our test conditions, histidine decreased slightly the catalytic effect of copper.

Dr. C. R. DAWSON: A number of years ago we were adding complexing agents to copper, in the presence of ascorbic acid, and found that with very minute amounts of protein the rate of oxidation was increased.

Mr. COLEMAN: What would be the approximate ratio of protein to copper in such a system?

Dr. C. R. DAWSON: I don't recall. However, as you increased the protein beyond a certain limit the reaction was inhibited.

Mr. COLEMAN: I should like to ask Dr. Dawson if he has any information regarding the mechanism of the ionic copper-catalyzed oxidation of ascorbic acid. Is the existence of an unstable intermediate ternary complex probable with inorganic catalysts?

Dr. C. R. DAWSON: Many investigators have studied the aerobic oxidation of ascorbic acid as catalyzed by cupric ions. All are agreed that hydrogen peroxide is formed. Dekker and Dickinson [*J. Amer. chem. Soc.*, 62: 2165 (1940)] have proposed a mechanism involving an intermediary semiquinone like ion and perhydroxyl, HO_2.

COPPER METABOLISM IN THE INVERTEBRATES

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The number of species of invertebrates which have been analyzed for the presence of copper since that element was demonstrated over one hundred years ago to occur in animals has become so great that a tabulation of the data from all sources would be prodigal to the extreme. Few forms from the Protozoa to the Arthropoda have escaped scrutiny (cf. the bibliography for major sources of data). Particular attention has been directed toward those species known to contain or suspected of containing the respiratory pigment hemocyanin. Yet despite the volume of analytical endeavor by chemical, histochemical, and spectrographic techniques, the significance of copper in invertebrate metabolism remains largely speculative. For the most part the data merely serve to strengthen the conviction that this element is universally present in the animal kingdom. That it is a normal rather than an adventitious component was early suspected from its widespread occurrence, a suspicion subsequently corroborated by studies on its accumulation and elimination.

In general, the invertebrates contain appreciably higher concentrations of copper than either vertebrates or plants. It has been in part this fact which has stimulated the numerous investigations of these phyla. Illustrative of the difference are the following averages calculated by Clare (19) from many sources and expressed as mg. per g. dry wt.: insects, 0.0919; marine invertebrates, 0.17361; vertebrates, 0.01196. Further calculations have also been undertaken by Clare in a series of unpublished papers in which are assembled data from many sources, together with his own extensive analyses. Comparisons of the frequency and distribution of an element are rendered difficult by the lack of uniformity on the part of investigators in expressing Cu content, as well as by variation in the degree of accuracy of the determinations. Add to this the fact that the copper content varies with age, individual, species, etc., and it is manifestly impossible from existing data to make worthy correlations with phylogeny, environment, or feeding habits. In this connection the only facts which stand out clearly are that arthropods and molluscs usually contain high concentrations whereas echinoderms, coelenterates, and sponges are usually very low (cf. Griffiths, 34; Dhéré, 28; Dubois, 29; Phillips, 58; Rose and Bodansky, 67; Severy, 68; Noddack and Noddack, 53; Webb, 75; Bertrand, 6; Clare, 19). Fewer data are available regarding annelids, and the data on tunicates are too incomplete to permit of comparisons.

It is obvious, however, that all of the forms studied usually contain greater concentrations than does the food that they ingest or the medium in which they live. From this it must be assumed that the element is concentrated within the body and maintained at some optimum level. These considerations lead naturally to the questions: which organs and systems are concerned with accumulation and elimination? what purpose does copper serve in the metabolism of the organism? and what effects ensue if copper balance is upset? It is proposed to discuss each of these in turn in so far as existing data permit.

Copper Balance

The phenomena of accumulation and elimination have been studied chiefly by two methods: (1) indirectly by differential analyses of various tissues and at successive developmental stages; (2) more directly by analyses of marine animals subjected to sea water containing high copper concentrations, and of terrestrial animals, principally insects, maintained on diets high in copper.

Marine Organisms. Differential estimations of the organs of Mollusca have showed highest concentrations in the hepatopancreas and gonads, with appreciable amounts in the mantle (McHargue, 46; McCance and Shackleton, 44; Paulais, 57). Lowest concentrations occur in muscular tissues (Phillips, 58; Cunningham, 23; Webb, 75). In the Crustacea, as exemplified by the lobster, highest concentrations were found in the stomach, digestive glands, liver, gonad, and eggs (McHargue, 46; Cunningham, 23; Clare, 19). The muscles and shell contain the lowest concentrations. The suggestion is implicit in these results that copper is absorbed primarily by way of the gut, and probably by the mantle also in molluscs, is accumulated in glands associated with the digestive system, and is eliminated by the general excretory organs. That which is not absorbed through the gut passes out in the feces. As to the marked amounts present in reproductive organs and their products, one can but speculate that this may represent an additional route of excretion (cf. Clare, 19) or a means of supplying the developing embryos with the requisite quantity of this element.

Of particular interest is the relation of copper to the propensity of oysters for becoming intensely green in certain localities. It has been shown that the green color of American oysters is concentrated in amoeboid cells which aggregate in cysts under the body epithelium and on the surface of the gills. There is some evidence that the intensity of green is proportional to the amount of copper. Galtsoff and Whipple (32) showed that normal oysters contain from 8.21 to 13.77 and green oysters from 121.71 to 271.91 mg. Cu/100 g. dry wt. (cf. also Willard, 77; Hiltner and Wichman, 38; Coulson et al., 22; McHargue, 46 for other values on normal oysters). They attempted to determine the exact nature and function of the pigment but were able to decide only that it was neither hemocyanin nor copper proteinate, that it exists in a highly dissociated state, and that there is no significant difference in the oxygen consumption of normal and green oysters.

The change in copper content with age and development exhibits different trends in different species. This is illustrated by three species studied by Marks (47). In the snail *Helix aspersa*, the copper per unit weight increases with increases in size of the animal; in the California sea mussel it decreases; in the octopus *Polypus bimaculatus* it remains constant. In any event, the experiments of Clark (20) proved that the maintenance of optimum copper levels is an active process. The soft tissues of barnacles noramlly contain much higher copper than sea water. In solutions of cupric citrate they absorb more than one hundred times the normal amount, a feat which dead barnacles are incapable of matching. When living specimens are replaced in fresh sea water, as much as two-thirds of the absorbed element can be eliminated. Mussels, by contrast, can eventually eliminate all of the excessively absorbed material and return to the original content of 0.0109 mg. Cu/g. dry wt.

Insects. Analyses by Melvin (48), Okamoto et al. (55, 56), Waterhouse (74), and Clare (19) have revealed that the distribution of copper in the tissues of insects follows essentially the same pattern as in the marine forms previously discussed. Thus the alimentary canal and malpighian tubules especially contain the largest amounts of this element, while the muscles, cuticle, blood, gonads, and foregut are poor in copper. A similar pattern is true of certain arachnids, e. g., the scorpion, and *Peripatus* (Clare, 19). The slime and crural glands of the latter contain the greatest amounts in its tissues. The outstanding difference between the terrestrial and marine forms is the relative amounts in the gonads.

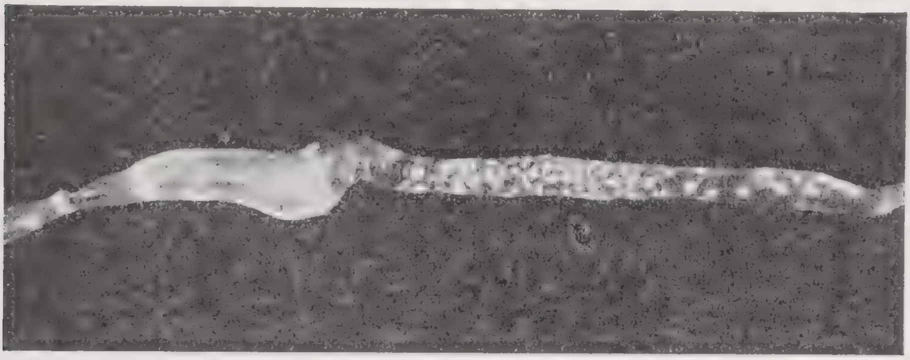

FIG. 1. Mid-midgut of the larva of *Lucilia cuprina* showing the darkly stained bands where copper is absorbed. Note the large flat unstained cells in the anterior zone. (From Waterhouse, 1945, by courtesy of the Commonwealth Scientific and Industrial Research Organization, Australia.)

Careful histochemical studies by Waterhouse (74) of the sheep blowfly, *Lucilia cuprina*, have shown that in the region of the larval mid-gut, within a zone having a pH of less than 5.3 (Fig. 1), there exist two bands of copper-absorbing cells. This region comprises about one-eleventh of the mid-gut. The cells of the malpighian tubules also exhibit typical staining reactions with carbamate, a fact which indicates the presence of copper here. Similar examinations of pericardial and ventral nephrocytes, muscles, foregut, and food gave negative results. Quantitative chemical determinations by the same author confirmed his histochemical studies (Table 1). It is noteworthy that the blood, although it forms 40 per cent of the live body weight, contains less than 14 per cent of the total body copper. The existence of special copper-absorbing cells in the mid-midgut of insects fed on a copper-enriched medium has also been confirmed by Poulson (60) in studies of forty species of *Drosophila.*

TABLE 1

Quantitative Determinations of Copper in the Tissues of *Lucilia cuprina*

Organ	mg. Cu/100 g. dry wt.
anterior and posterior midgut	8.7
ant. and post. midgut and hindgut	7.5
mid-midgut	22.0
hindgut	13.1
entire gut	10.3
cuticle	2.0
fat body	3.2
cuticle and fat body	2.4
blood	2.2
whole larva	2.9

The cells give a characteristic orange-red fluorescence in ultraviolet light. A similar fluorescence is given by livers of a number of crustacea and cephalopods from which a fluorescent heat-stable copper-containing material has been isolated.

The implication of the foregoing data that certain cells of the midgut are selectively concerned with the absorption of copper and that the malpighian tubules are concerned with its elimination is borne out by the ontological history of the distribution of copper and by the reactions of the various organs to high copper diets. The data of Waterhouse are given in Table 2 by way of illustration. This picture is in substantial agreement with Akao's (1) data on the silkworm and Clare's (19) on various other species. It differs in one respect from the results of Melvin (48) and Clare (19). These two workers found that cast skins, exuviae, and egg cases contain appreciable amounts. This was not true of the cast *Lucilia* puparia. All authors agree, however, that the egg shells contain more copper than the insect which produces them or emerges from them. Waterhouse explained this as probably due to the loss of water, carbon dioxide, and other substances during development and the actual hatching process. Certainly the amount is approximately the same in the egg, newly hatched larva, and adult. Early in larval development there is a drop followed by a rise. During this period copper is clearly accumulated from the food. After feeding ceases (last instar and prepupa) the rise continues (Fig. 2).

TABLE 2

THE DISTRIBUTION OF COPPER IN THE DIFFERENT DEVELOPMENTAL STAGES OF *Lucilia cuprina*

Stage	mg. Cu/100 g. dry wt.
egg	5.9
egg shells	13.6
1st instar unfed larva	7.0
larva after 0.5 days	3.0
larva after 1.5 days	2.3
larva after 2.5 days	2.9
larva after 3.5 days	3.2
prepupa	3.5
pupa	3.9
empty puparia	4.0
unfed adult	6.2

This is explained by Waterhouse as due to copper retention during the utilization of stored food in metabolism.

The concentration of copper in the tissues is directly dependent upon its presence in food. All species placed on high copper diets accumulate copper above the normal value and then lose it again slowly. The roaches *Periplaneta americana* and *Blatta germanica*, for example, show a fivefold increase when placed on a high but sub-lethal diet (Clare, 19). Those on a fasting diet (water only) fall to 15 to 20 per cent below normal. Although copper increases in all tissues which normally contain it, the increase is proportionally greater for those with high copper than those with low, so that the order of content of the tissues remains the same. Nevertheless, the increase in such tissues as cuticle, muscles, and fat body indicates that more is absorbed into the hemolymph and distributed to these tissues. Waterhouse has shown, however, that the increase is the same on diets containing 1 mg. of added Cu as on 2 mg. Therefore, there must be some regulating mechanism in operation.

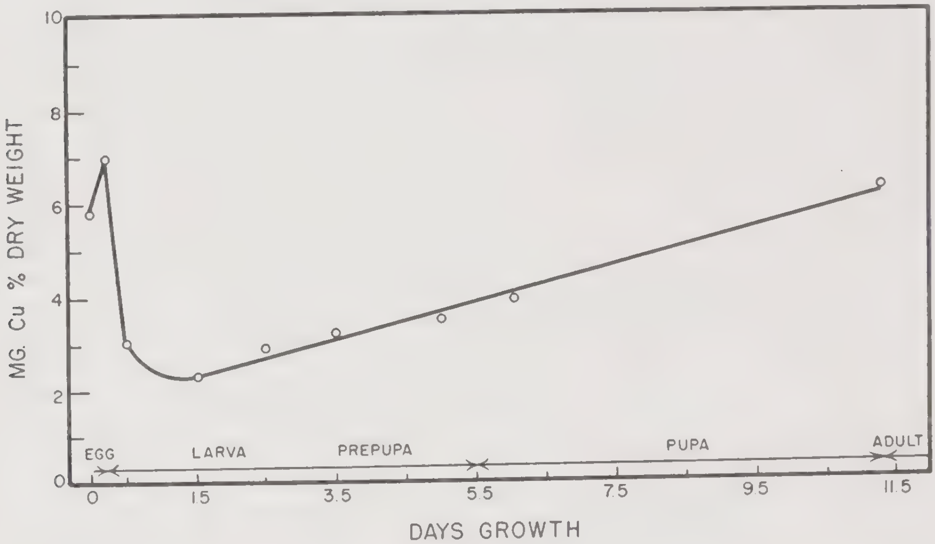

FIG. 2. Copper content of *Lucilia cuprina* at different stages of development on enriched medium at 30°C. (Redrawn after Waterhouse, 1945, by courtesy of the Commonwealth Scientific and Industrial Research Organization, Australia.)

All of the accumulated evidence indicates (1) that copper is absorbed by the midgut, where its presence can be demonstrated for a time as ionized copper; (2) that it is distributed by the hemolymph and later enters into organic combination, in which state it is no longer demonstrable by staining; (3) that the malpighian tubules take up increased amounts as a result of greater amounts in the blood when the insect is on a high diet; (4) that the malpighian tubules are able to regulate the amount of copper up to a certain point, after which their capacity for elimination is overtaxed and toxic symptoms arise; and (5) that in the prepupal stage any excess copper which has accumulated during larval life is mobilized for elimination either by way of the malpighian tubules or by way of the degenerating midgut (cf. also Liozin, 43—increase in feces reaches a maximum at time of cocoon spinning). Clare has also advanced the suggestion that the high content in cast cuticles, etc., observed by Melvin and himself, represents a type of storage excretion.

The fact that copper balance is so carefully regulated in all of the invertebrates investigated supports the conviction that this element is essential to the organism. Analyses suggest that a large percentage of it is firmly bound in organic combination. In the blowfly, for example, Waterhouse has demonstrated that at least one-third of the total concentration is in this form. It is not surprising, then, that its importance in at least two metabolic roles has been confirmed by the early demonstration of its presence in hemocyanin and the more recent proof of its presence in tyrosinase. That it may also bear some relation to iron metabolism, here as in vertebrates, has been suggested, but nothing is known of the relation (cf. Elvehjem, 30). For a discussion of hemocyanin the reader is referred to Redfield's (66) review and Chapter 8.

Copper Protein Oxidases

On the basis of a variety of phenolic compounds which could serve as chromogen substrates in vitro for enzymes which catalyze the formation of melanins, early investigators named as occurring in invertebrates such enzymes as dopa oxidase, catechol oxidase, diphenolase, and polyphenolase. As Sussman (69) has observed in his extensive review of the functions of tyrosinase in insects, it is now generally conceded that these should be considered as belonging to the tyrosinase complex. Such obviously is the case with protyrosinase, an inactive form of tyrosinase described by Bodine et al. (11) as occurring in the eggs of grasshoppers and mealworm larvae. None the less, as Sussman has also pointed out, there is evidence that other copper protein oxidases may also occur, at least in insects, although scarcely any attention has been paid to these.

Since copper proteins have been considered at length in Chapters 1 to 5, the present discussion will be restricted to directing attention to a few of the more important functions of these enzymes in invertebrate physiology. Specifically, they are concerned (1) with hardening of the cuticle of organisms which molt the exoskeleton at successive developmental stages or which deposit eggs encased in shells or oothecae of similar materials; (2) with mechanisms at attachment of marine organisms that become sessile after a transitory free-swimming larval stage; (3) with pigment and pattern formation (see Sussman, 69 and Chapter 3) and the production of melanin in the ink of the octopus (Cunningham, 23); and (4) with injury and blackening of the blood.

Of these divers functions, that of tanning has attracted most attention. As is well known, the cuticle of freshly molted insects is soft and colorless initially but rapidly darkens and hardens. Pryor (61) was the first to prove that these changes in the ootheca of the cockroach and the cuticle of the blowfly larva result from the quinone tanning of a protein. The tanning involves introduction of primary valence cross-linkages into the protein network as a result of combination with a quinone derived from phenolic substances present in the blood. Dennell (26) has demonstrated that these phenols arise from the oxidation of blood tyrosine by tyrosinase (cf. also Trim, 70 and Fraenkel and Rudall, 31).

But quinone tanning is by no mean confined to the insects. It is now recognized by many that the cuticle of crustaceans is very similar to that of arthropods both structurally and chemically. Pinhey (59) had shown that the blood of certain species contains tyrosinase, and Dennell (27) demonstrated that quinone tanning similar to that occurring in insects takes place in the exocuticle of crustaceans.

The commonness of tanning in invertebrates was emphasized by Brown (14), who developed the idea that among the characteristics required of animals for colonization of the land was a skin in which the proteins could be dried and impregnated with lipids without undergoing any pronouncing changes in mechanical properties. Vertebrates developed keratin, a protein in which molecules are cross-linked by cystine bridges. Invertebrates, on the other hand, possess skeletal proteins of the collagen type, and tanning is necessary to render a cuticle of this kind hard and impervious. Evidence of the existence of quinone-tanned proteins has been found (Brown,

15) in the central capsule of a marine protozoan (*Thalassicola*), in the external cortical layer of the cuticle of the parasite *Ascaris lumbricoides*, in the chaetae of a polychaete worm (*Aphrodita*), in the egg cases of the liver fluke (*Fasciola hepatica*), in the byssus of the mussel (*Mytilus edulis*), in myriopod cuticle (Blower, 10), and in the cocoon shell of *Dendrocoelum lacteum* (Nurse, 54). Available data indicate, however, that the sequence of tanning events is not identical in all forms (see also Burton and Stoves, 16). The evidence relating to the byssal threads of the mussel confirms a suggestion made earlier by Pyefinch and Downing (64) that the cement used to attach a number of sedentary marine animals to the substratum is formed from a mucoprotein basis which subsequently undergoes quinone tanning.

Attempts have been made to show that tyrosinase is of major importance in insect metamorphosis and also as a terminal oxidase in the respiration of intact animals. These hypotheses have not been supported by recent data (see Sussman); however, it is indicated that tyrosinase does increase the rate of oxygen uptake in damaged tissues. Proposals have also been advanced by several investigators (e. g., Henke, 37; Cordier, 21; Bhagvat and Richter, 7) that tyrosinase in the hemolymph and corpuscles of various insect and crustacean species may, by acting upon the phenolic products of protein breakdown, assist in the removal of these toxic materials.

Growth and Metamorphosis

Less well authenticated than the roles of copper as reviewed above is its part as a necessary element for the normal growth and development of animals. Most of the available data relating to unicellular forms indicate that it is not a necessary constituent. Bowen (13) has reported, for example, that copper is not necessary for normal growth in *Chilomonas paramecium.* Yet it should be remembered that one of the difficulties attendant upon studying growth requirements in this respect is the practical impossibility of completely depleting an animal of its supply of copper. Since many metazoans derive a large initial supply from the egg, experiments would have to be run over an unknown number of generations in order to make inroads on the bound copper.

Although there are no records of untoward effects produced in insects as a result of continued feeding on a copper-deficient diet, curious results have been obtained by feeding certain phytophagous species on plants grown on copper- or zinc-deficient media. Caterpillars of *Alabama argillacea*, for example, suffered a mortality rate of 90 per cent when grown on Cu-deficient plants as compared to a rate of 8 per cent in controls (Creighton, 23). Decimation was greatest during the first and second stadia. Survivors turned an unnatural brown, and the length of larval life was increased from 31.3 days to 38.8 days. Since in species of pantophagous and necrophagous insects which have been raised on artificial copper-deficient media no such drastic effects have been observed, there is some reason to suspect that the absence of copper may have acted only indirectly. This has been known to happen in reverse instances where excess copper has been applied to food plants. Bordeaux mixture and other copper compounds applied as sprays to bean and potato plants to control the potato leafhopper (*Empoasca fabae*) were observed to have no killing effect. Three to five days later effects appeared in insects and continued as eggs hatched and the newly emerged larvae continued to feed. Only within those areas to which the spray had been applied was it effective. It has been claimed that the plant is induced to produce a toxic material as a result of metabolic alterations following the application of copper (DeLong, 25).

Thus it has not been possible to show by withholding copper that it is necessary for normal growth. The converse approach of exposing animals to high concentrations in an effort to accelerate growth has also given negative results (cf., e. g., Miller, 49). On the other hand, there is some evidence that increased copper affects development in that it may induce settling and attachment of marine organisms and accelerate their metamorphosis. Prytherch (62, 63) has maintained that larvae of the American oyster (*Ostrea virginica*) are stimulated to settle on the substratum and metamorphose into a spat by increased concentrations of copper released into estuaries at low water stages by river water. The experiments and conclusions of Prytherch have, however, been severely criticized by Korringa (42), who worked with *Ostrea edulis* but also cited other work on the American oyster in support of his views. For other marine forms there is more conclusive evidence that attachment is indeed stimulated by copper. Pyefinch and Downing (64) stated that median sub-lethal concentrations of copper, mercury, benzoquinone, and ascorbic acid induce attachment of the actinula of *Tubularia larynx*. These compounds apparently cause a decrease in the rigidity of the aboral tentacles and so allow the animal to come into contact with the substratum prematurely. Small concentrations of copper are reported to stimulate attachment in *Bugula neritina* (Miller, 49). Clark (20) has observed that 0.06 mg. Cu/l. accelerates development in barnacle cyprids after they have metamorphosed. Grave and Nicoll (33), in a lengthy study of *Ascidia nigra* and species of *Polyandrocarpa*, have found that metamorphosis in these forms can be greatly accelerated. Here metamorphosis may be thought of as a sudden breakdown of the larval action system which releases the ascidiozooid from an inhibitory influence. A resumption of growth and differentiation of the action system of the ascidiozooid then follows in sequence. Under normal conditions a period of thirty to forty-eight hours must elapse after hatching before 100 per cent of the larvae metamorphose. Sea water extracts of pharyngeal, atrial, or mantle tissues of adults will induce complete metamorphosis three hours after hatching. Other accelerators include: L-histidine, leucine, glycine, cysteine, D- and L-alanine, and copper salts. Non-accelerators include: tryptophane, tyrosine, diiodotyrosine, L-aspartic acid, D-glutamic acid, D- and L-phenylalanine, D-lysine, and cystine. Of all accelerators copper salts are the most active. At concentrations greater than 5×10^{-4}M they are toxic, at 5×10^{-4} to 4×10^{-6}M the disruptive phase of metamorphosis and partial retraction of the tail is accelerated but subsequent growth is stopped, at 2.5×10^{-6} to 1×10^{-6}M metamorphosis is accelerated and growth follows immediately, at concentrations below 8×10^{-7}M metamorphosis is inhibited. Despite the fact that copper and adult tissue extracts both accelerate and that copper can be demonstrated in these tissues, there is no good correlation between the percentage of copper in the different types of tissue and their effectiveness in accelerating metamorphosis. Glaser and Anslow (32a) have recently reinvestigated this problem and presented a theory to explain metamorphosis in terms of copper activation and inhibition of enzyme systems. They suggested that copper first inactivates the larval oxidation-reduction system. Morphological disintegration results. Rudiments which remain and have thus far been inhibited are now activated so that constructive metamorphosis commences. The mechanism of inhibition involves either copper starvation due to competitive absorption by larval catalysts and substrates, or acquisition and storage in early development of an excess of copper. Activation is initiated by a return of copper in the rudiments to optimum concentration.

Toxicity

When the copper concentrations to which an organism is exposed are increased beyond the capacity of the excretory organs to eliminate the material, death ensues. While the physiology of copper metabolism and its mode of action as a poison are even now not completely understood, it has been realized for a long time that copper compounds are effective killing agents. One of the earliest effective insecticides, Bordeaux mixture, contained copper compounds, and copper in combination with arsenic is still in widespread use.

The mode of action of copper insecticides has never been elucidated. Waterhouse in his study of the blowfly found that as more and more copper was added to enriched medium, growth as determined by live weights was progressively decreased (Fig. 3). If, in this experiment, copper was added to a basal medium (which contains agar in place of egg white) rather than to the enriched medium, growth retardation was severe. It is clear that some factor other than copper concentration was responsible, and it appears that this may have been the paucity of protein in the basal medium. It is probable that the toleration of high copper concentrations in the enriched medium was possible because of the formation of copper protein compounds.

There is also an indication that copper may be concerned in cyanide poisoning, at least in citrus scale insects. Sometime around 1922 a strain of scale appeared that was resistant to cyanide fumigation. Haas (36) initiated a thorough investigation of the chemical composition of the resistant and normal susceptible strains in an endeavor to understand the nature of the resistance. The most outstanding difference in the two strains was the amount of copper present, the resistant strain having only one-fifth to one-tenth as much as the susceptible.

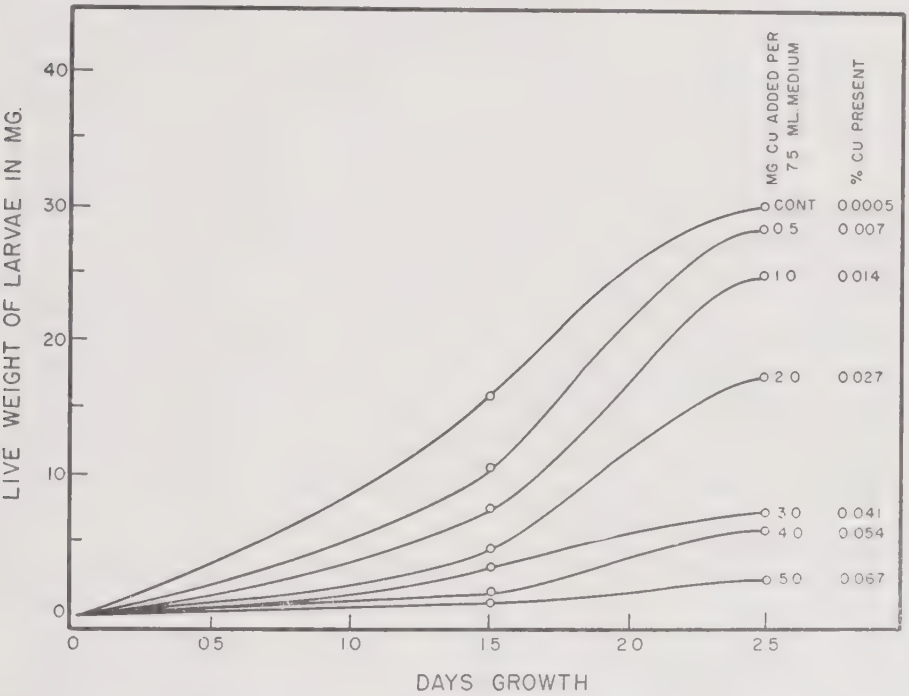

Fig. 3. Growth curves of larvae of *Lucilia cuprina* on enriched medium containing added copper. (Redrawn after Waterhouse, 1945, by courtesy of the Commonwealth Scientific and Industrial Research Organization, Australia.)

Excessive but sub-lethal amounts of copper in the diet may induce marked morphological anomalies. Such has been shown to be the case with the American and German roaches (Clare, 19). The wings present a curled appearance and are more brittle than normal. This is interesting in view of abnormalities reported in marine organisms subjected to high copper concentrations. The roaches as well as the blowfly also exhibit a marked increase in water content under these conditions.

Interest in the toxic action of copper on marine organisms has centered around its use as an antifouling device. Many workers, chief among whom are Miller (49), Clarke (20), Barnes and Stanbury (5), Pyefinch and Mott (65), Weiss (76), Hunter (39), and Woods Hole Oceanograph. Inst. (78), have concerned themselves with investigations of the action of copper and other metals in preventing the attachment of marine organisms to the hulls of ships. For a more complete treatment of this subject the reader is referred to (78).

Barnacles exposed to copper or zinc exhibit malformations may be indicative of interference with calcium metabolism (Weiss, 76). On certain paint surfaces or as a result of crowding or other factors the margin of the shell becomes deeply scalloped, suggestive of localized interruptions of marginal growth. The bases of the barnacles are characterized by increased opaqueness of the radial canals, possibly as a result of increased $CaCO_3$ deposition. Different species show the same order of degree of malformation as susceptibility to copper poisoning. The condition is especially characteristic of copper poisoning and is not found following exposure to lead or mercury.

In an attempt to elucidate more fully the action of heavy metal poisoning, Jones (40) investigated the effects of ionic copper on the oxygen consumption of *Gammarus pulex* and *Polycelis nigra.* The first response to $CuSO_4$ is a slight rise in respiration, presumably a result of inhibition of ciliary locomotion with a consequent increase in muscular activity (cf. Hunter, 39). Comparisons with decreases of respiration caused by sub-lethal cyanide suggest that the decrease is not in itself the cause of death. The author believes that his results are consistent with the interpretation that copper penetrates cells and precipitates the cytoplasmic proteins as Cu-proteinates, with gradual complete destruction of the protoplasm. In the case of barnacles and mussels, Clarke (20) suggested that death results from impairment of some function, such as feeding, due to a general depressant action rather than to any specific action on " some vital substance." Hunter, too, felt that his data on toxic effects in crustaceans supported the idea that copper acts somewhat indirectly by upsetting the equilibrium of some undetermined system. Miller's (49) data tend to favor the hypothesis that copper inactivates essential enzymes. Chalkley and Voegtlin (17), in a series of studies of cell division in protozoa, found that low concentrations of copper salts inhibit cell division in *Amoeba proteus* and that small amounts of glutathione increase it.

At this time one can only conclude that far too little is known of the mechanism of action of heavy metal poisoning in general to permit of satisfactory explanation of the results derived from studies of the Invertebrata.

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DISCUSSION

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Dr. DETHIER: I believe the paper to which you refer is the only extensive investigation concerning vanadium in the tunicates.

Dr. BEAR: Do you have any knowledge of the earthworms?

Dr. DETHIER: The annelids have been greatly neglected. Those that have been most extensively studied are the marine forms, and there only a cursory examination has been made. I know of no investigation of the earthworms where the data are not suspect because of the analytical methods that were used at that time.

Dr. POULSON: I would like to ask Dr. Dethier about the excretion by the malpighian tubules. Our observations would suggest that there is very little accumulation of copper in the malpighian tubules beyond a certain level; all the rest is accumulated in the copper cells of the gut.

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Dr. POULSON: We have all depended upon Waterhouse's experiments as a guide. We have observed with *Drosophila*, when histochemical methods are used, that there is no correlation between the intensity of staining in the malpighian tubules and the amount of copper fed. On the other hand, there seems to be a good correlation between the intensity of staining of the mid-gut cells and the amount of copper fed. My interpretation of these results would be that the mid-gut cells represent the chief organs of accumulation and excretion.

Dr. DETHIER: You visualize this, then, as storage excretion until the advent of pupation?

Dr. POULSON. Yes, I think that is true. It remains through metamorphosis to the emergence of the adult. Waterhouse's observations and mine on iron and copper and the findings of Bowen on manganese all implicate the mid-gut epithelium as the principal site of mineral excretion. Experiments utilizing radioisotopes carried out by Dr. V. T. Bowen and myself make it reasonably certain that the malpighian tubules are not primarily concerned in the excretion of these elements. The copper of malpighian tubule cells is interpreted as an integral part of enzymes present there.

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HEMOCYANIN

Albert C. Redfield
Woods Hole Oceanographic Institution and Harvard University

The hemocyanins appear to be the first copper compounds to have been discovered as normal constituents of living organisms. Their occurrence in the blood of many of the larger invertebrates and their striking blue color invite attention. Their availability in quantity and the fact that they occur in solution in the circulating fluids facilitate a study of them. Their function in the transport of respiratory gases lends itself to investigation. Finally, the presence of copper in the hemocyanin molecule provides a convenient basis for exactly apprehending the chemical relations of their attributes. As a result, the hemocyanins may be listed among the better known proteins and are the only class of such copper compounds to have received extensive systematic study, either as to their chemical characteristics or their physiological function.

The blue color of molluscan blood was noted in the seventeenth century by Swammerdam. The presence of copper in this blood was detected in 1847 by Harless and Bibra, and Paul Bert first recognized the relation of the blue pigment to oxygen transport (20). The names hémocyanine and oxyhémocyanine were coined by Fredericq, who made the first study of its properties (12). As the physical chemistry of hemoglobin became better known and its relation to function was understood, in the early decades of this century, interest in hemocyanin was stimulated. Four substances were known which appeared to function in respiratory transport: hemoglobin, chlorocruorin, hemerythrin, and hemocyanin. All are metallic protein compounds, but the latter, unlike the others which contain iron as the basis of their functional peculiarity, made use of copper. It was a question whether these substances shared the unique properties of hemoglobin and functioned in accordance with the same general physical-chemical principles.

We can now affirm that these proteins have entirely analogous physical-chemical properties that enable them to perform their function in the transport of oxygen and carbon dioxide. Each of the four named classes is distinguished by well defined characteristics of chemical composition—such as the porphyrin peculiar to chlorocruorin, or the copper of the hemocyanin molecule. Within each class the respiratory proteins of different kinds of animals are distinguished in more subtle ways, which cannot be defined in terms of composition but show themselves in quantitative behavior (11, 25). The hemocyanins, then, are a group of proteins similar in containing copper and in binding oxygen in the labile fashion required for respiratory transport, but displaying among themselves distinct specific differences.

The known distribution of hemocyanins in the animal kingdom is scattered and limited. They are found in two phyla, the molluscs and the arthropods. Among the former, they occur in the cephalopods and gastropods, but have not been identified in any other classes. Among the latter, they appear to be limited to the decapod crustaceans (crabs and lobsters), the Xiphosura (the horseshoe crab, *Limulus*), and the scorpions. Among these organisms the hemocyanin occurs in solution in the blood or body fluid; it has never been found in corpuscles or within tissue cells (26).

The hemocyanins are soluble in dilute salt solutions, but if the concentration of electrolyte is sufficiently low they are insoluble at the isoelectric point. They are thus classed among the globulins. The units which each contain one atom of copper have molecular weights of 25,000 to 37,000. Since two atoms are required to bind one molecule of oxygen, the minimal molecular weight of the functional unit is 50,000 to 74,000. These units undergo reversible aggregation under the influence of the chemical environment and exist as giant molecules having molecular weights ranging from 400,000 to 10,000,000 (9). The largest aggregates contain up to 400 atoms of copper, and each aggregate may bind as many as 200 molecules of oxygen. The diameter of the molecule, as determined with the electron-microscope is 8 mμ for squid hemocyanin and 17 mμ for *Limulus* (5). The hemocyanins are exceeded only by the viruses in molecular size.

The value of these large aggregates of hemocyanin to the organism appears to be that they permit large quantities of the respiratory protein to exist in the blood, and thus provide for a high oxygen capacity, without unduly altering the osmotic pressure and other properties of the circulating fluid (29). The aggregate thus serves a function similar to that of a red blood corpuscle (10). Perhaps it represents the limit of size which has stability without developing the heterogeneity of structure displayed by true corpuscles and cells.

In the more active organisms, such as the cephalopods, the hemocyanin present in the blood enables it to combine with 4 or 5 ml. of oxygen per 100 ml. of blood. This is sufficient to be of advantage in respiration. It has been shown that the blood undergoes oxygenation and reduction in the cycle of circulation, and that death from asphyxia by CO_2 is related to the failure of the blood to become oxygenated (27). In the less active species, the concentrations of hemocyanin are smaller and it is not completely certain that they function in respiratory transport.

The pressure at which hemocyanin dissociates from oxygen varies widely in different species, as do the conditions under which the pressure contributes to the transport of carbon dioxide. On the whole, these properties appear to be related to the respiratory problems with which the different kinds of animals are confronted.

The development of knowledge and thought about the copper-proteins is following closely a pattern set by the study of hemoglobin. Like hemocyanin, hemoglobin was found to be the respiratory pigment in a limited number of phyla scattered through the animal kingdom. It occurs in vertebrates generally, in many annelids, and in isolated cases among echinoderms, molluscs, and insects. It was also found to be a constituent of muscle cells in many species. This distribution did not appear to make sense according to any scheme of evolution. Moreover, there did not seem to be any clean-cut rule as to which organisms had hemocyanin and which hemoglobin. Some snails used one of these pigments, some the other. One species, *Busycon*, employs hemocyanin as a respiratory pigment, yet has hemoglobin in some of its muscles (3).

With the discovery of the cytochromes by Keilin and the subsequent identification of catalase, peroxidase, and indophenol oxidase as heme compounds, it has become evident that the hemoglobins may be looked upon as but one of a large class of substances employing a common atomic configuration, heme, as the basis for the development of their special activities. To quote Anson and Mirsky:

> What is clear now is the possibility on the basis of a single haem, of a variety of haem pigments having very different properties in adaptation to very different needs. . . . It is no longer necessary to assume a "special creation" for each hemoglobin or hemochromogin. (1).
>
> Hemoglobin, it now appears, may no longer be regarded as the preeminent heme pigment, nor is its haphazard distribution now so mysterious. Hemoglobin is merely an occasional specialized derivative of an iron substance, heme, which is much more widely distributed. Similarly, the nucleus of the rare chlorocruorin, or a heme very like it, is present in aerobic cells generally. It would be interesting to know whether hemocyanin, the copper pigment which carries oxygen in the bloods of some crustacea, is also an occasional derivative of a universal copper compound. (2).

It now seems as though the latter speculation may be justified. We can list three oxidative enzymes, and two other cellular constituents, not yet shown to be active substances, as copper proteins. These substances resemble hemocyanin closely in copper content and in other ways. Moreover, hemocyanin itself acts weakly as a pseudophenolase and thus appears to be catalytically active (4).

The most careful comparison of a copper enzyme with hemocyanin has been made by Kubowitz (16) in a study of polyphenol oxidase. A resemblance was found in their copper content, which was 0.19-0.20% for the enzyme compared to a range of 0.17-0:26% for various hemocyanins. When exposed to carbon monoxide in the presence of a suitable substrate, polyphenol oxidase forms a compound containing one molecule of CO for each 2 atoms of copper. This is the ratio in which hemocyanin combines with CO and with oxygen. When exposed to potassium cyanide, the enzyme and hemocyanin alike split into a protein and a copper salt. After this, the enzyme protein is no longer catalytic, and the hemocyanin can no longer bind oxygen. If the cyanide is removed by dialysis and a copper salt added to the protein, the enzyme regains its activity and the hemocyanin can once more combine with oxygen. On the basis of these observations, Kubowitz concluded that the copper group is the working group of both these substances. Dissimilarity between them was shown by the facts that the enzyme, unlike hemocyanin, does not combine with oxygen and does not have a blue color. Furthermore, after treatment with cyanide, copper can be restored to hemocyanin only if it is supplied in the cuprous state.

The other copper proteins, which include the enzymes laccase and ascorbic acid oxidase and the inactive substances hemocuprein and hepatocuprein, are blue or blue-green in color like hemocyanin. The similarity of their copper content is striking, as may be seen from Table 1. There has been some discussion as to whether the differences reported are real, or are due to differences in purity of the preparations. However this may be, the spread of the measurements is not greater than that unquestionably demonstrated for the hemocyanins of different species.

The central problem in the comparative chemistry of the copper proteins is to discover whether copper is bound into a complex of atoms with some common configuration which serves as the basis for the specific activities of these proteins. We may call such a complex a prosthetic group. In the case of the iron proteins, we know that a prosthetic group exists in the porphyrin-iron complex heme. In the case of the copper proteins, we know that specific activities, associated with the presence of copper, cannot be attributed to the properties of copper alone but must reflect new properties which depend upon associated structure. Because of their availability in quantity, the hemocyanins are the convenient material on which to study the nature of the complex which contains the copper.

In spite of nearly fifty years of study, little progress has been made in understanding the character of the complex to which copper is linked. Early workers showed that the copper was easily separated from the protein by acid and concluded that it was bound as an albuminate. These observations, together with the later experiments with cyanide, show at least that the linkage is a labile one. Differences in the ease with which copper may be separated from different hemocyanins is indicated by the fact that the hemocyanins of *Octopus*, *Limulus*, and *Busycon* give the biuret reaction on the mere addition of alkali, but this is not true of the hemocyanin of *Helix* unless a trace of copper is also added (26).

TABLE 1

ELEMENTARY ANALYSES OF COPPER PROTEINS

	Nitrogen	Sulphur	Copper	Reference
	per cent	*per cent*	*per cent*	
HEMOCYANINS				
Gastropod—*Helix*	15.15	0.76	0.24	(26)
Busycon	15.9	1.23	0.245	(26)
Cephalopod—*Octopus*	15.9	1.04	0.25	(26)
Loligo	15.75	1.19	0.26	(26)
Xiphosura—*Limulus*	16.9	1.10	0.173	(26)
Crustacea—*Homarus*	16.78	0.90	0.187	(26)
Jasus	.....	.....	0.148	(21)
HEMOCUPREIN				
Mammalian erythrocytes	14.35	1.12	0.34	(17)
HEPATOCUPREIN				
Ox liver	.....	.....	0.25	(17)
TYROSINASES				
Potato polyphenol oxidase	14.4	.....	0.20	(16)
Mushroom—*Psalliota*	.....	.....	0.3	(14)
Mushroom—*Lactarius*	.....	.....	0.23	(8)
LACCASE				
Rhus species	.....	.....	0.24	(15)
ASCORBIC ACID OXIDASE				
Summer squash	.....	.....	0.24	(19)

A number of derivatives containing copper have been obtained from the hemocyanins of different species by treatment with strong alkali or with acetone and acid. Hematocyanine obtained from *Helix* hemocyanin, using the methods employed in separating the phyllins of chlorophyll, was found to contain 7 per cent copper and to yield a test for pyrrol, but gave no evidence of porphyrin structure. Hämocuprin, which should not be confused with the hemocuprein of Mann and Keilin, is a product which was obtained from *Octopus* hemocyanin by Schmitz, and which has 6.27 per cent copper. It was thought to be a tribasic organic acid containing copper in complex binding. Although Schmitz found no evidence of a porphyrin structure in hämocuprin, a similar product obtained by Laporta appeared to contain pyrrol (26).

An unnamed derivative prepared by Conant and Humphreys from *Limulus* hemocyanin contained 21.5 per cent copper, which was practically all that was available in the parent protein. This product was believed to be in reality the naturally occurring prosthetic group and not a recombination of fragments of the original molecule. It gave no evidence of containing pyrrol nuclei. It appeared to be a complex copper salt of an unknown sulphur compound and a polypeptide that consisted of one molecule each of leucine and tyrosine and three of serine. The sulphur compound appeared to be linked through the copper, and not by a peptide linkage to the polypeptide (7). A somewhat similar preparation has been made from hämocuprin which was obtained from the crab, *Cancer pagurus*, and from *Helix*, and which contained tyrosine, leucine, and arginine, but no sulphur (30).

It seems certain that the prosthetic group of hemocyanin is not a porphyrin derivative and that the copper is closely associated with a complex of polypeptide nature. The derivatives containing copper which have been obtained seem more like fragments of the protein molecule than like independently stable structures such as the heme of hemoglobin (30). Failure to isolate such a stable complex does not, of course, preclude the existence of some highly specific arrangement of atoms in association with copper which may underlie the activity of the copper protein, yet fail to resist the conventional procedures of isolation. A recent suggestion is that no separate prosthetic group is present, but that "specific orientation of the protein molecules enables the copper atoms to become activated for combination with molecular oxygen" (22).

If the methods of organic chemistry have not been wholly successful in dealing with the prosthetic group of hemocyanin, we may nevertheless define some of its properties and its reactions to the protein molecule as a whole by more general considerations. The copper itself appears to be in the cuprous condition, and remains so when the hemocyanin combines with molecular oxygen. The copper may be oxidized, forming methemocyanin, by sufficiently strong oxidizing agents. The oxidation-reduction potential of this system is about +0.55 volt. The oxidation involves one hydrogen equivalent for each atom of copper in the hemocyanin. The methemocyanin retains the ability to combine reversibly with molecular oxygen (6).* The failure of cupric ions to combine with hemocyanin, from which copper has been removed with cyanide, is consistent with the cuprous nature of copper in hemocyanin.

The combining ratios of hemocyanin and oxygen, or carbon monoxide, indicate that two atoms of copper are required to bind one molecule of gas. This is true of all species which have been studied. The implication is that the prosthetic group contains two atoms of copper in close association (26).

The equilibrium of oxygen with hemocyanin, described by the so-called oxygen dissociation curve, indicates that several of the prosthetic groups of each molecule may become associated so as to react as units in combining with oxygen (28). In a study of hemoglobin, it was pointed out by Hill (13) that in accordance with the mass law the fraction of the respiratory protein oxygenated, y, at any oxygen pressure, x, is given by the expression

$$y = \frac{Kx^n}{1 + Kx^n},$$

where K is the equilibrium constant and n is the number of prosthetic groups so associated as to form a stable oxygenation product. The dissociation curve of the purified hemocyanin of *Limulus* and of the gastropod *Busycon* are accurately described by this equation, taking $n = 1$ (Fig. 1). This implies that the prosthetic groups are acting independently, as though each were a separate molecule.

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* The conclusions of Conant, Chow, and Schoenbach (25) on which these statements are based have been questioned by Rawlinson (22).

The oxygen equilibrium in the native serum shows great variety, both in the form of the dissociation curve and in the pressures at which oxygen combines with the hemocyanin. At certain pH values, the equilibrium for selected species of hemocyanin conforms to Hill's equation, taking $n = 2$ (Fig. 2, F, G, and H) or $n = 4$ (Fig. 2, B and I). This implies that the prosthetic groups are associated in pairs or in groups of four and that each association acts as a unit which must be completely oxygenated in order to form a stable product.

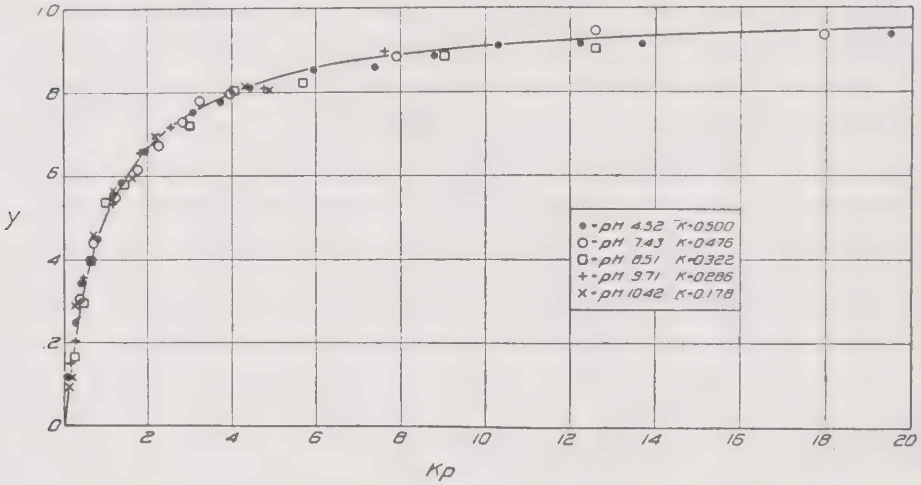

FIG. 1. Oxygen dissociation curve of purified hemocyanin of *Limulus polyphemus*. Ordinate, *y*, the fraction of hemocyanin present as oxyhemocyanin. Abscissa is the product of the oxygen pressure, pO_2, and the oxygen equilibrium constant, *K*. The curve is drawn in accordance with the mass law assuming n equals 1. From Redfield (24).

Under most conditions, the dissociation curves (Fig. 2, C, D, and E) cannot be described by Hill's equation. A satisfactory " fit " can be obtained, however, by an equation based on the assumption that the prosthetic groups may be associated in aggregates of 1, 2, or 4, and that such aggregates are present in proportions which vary with the hydrogen ion concentration (Fig. 3). When analyzed in this way, the constants expressing the proportions of each type of aggregate and their oxygen equilibrium constants vary in a

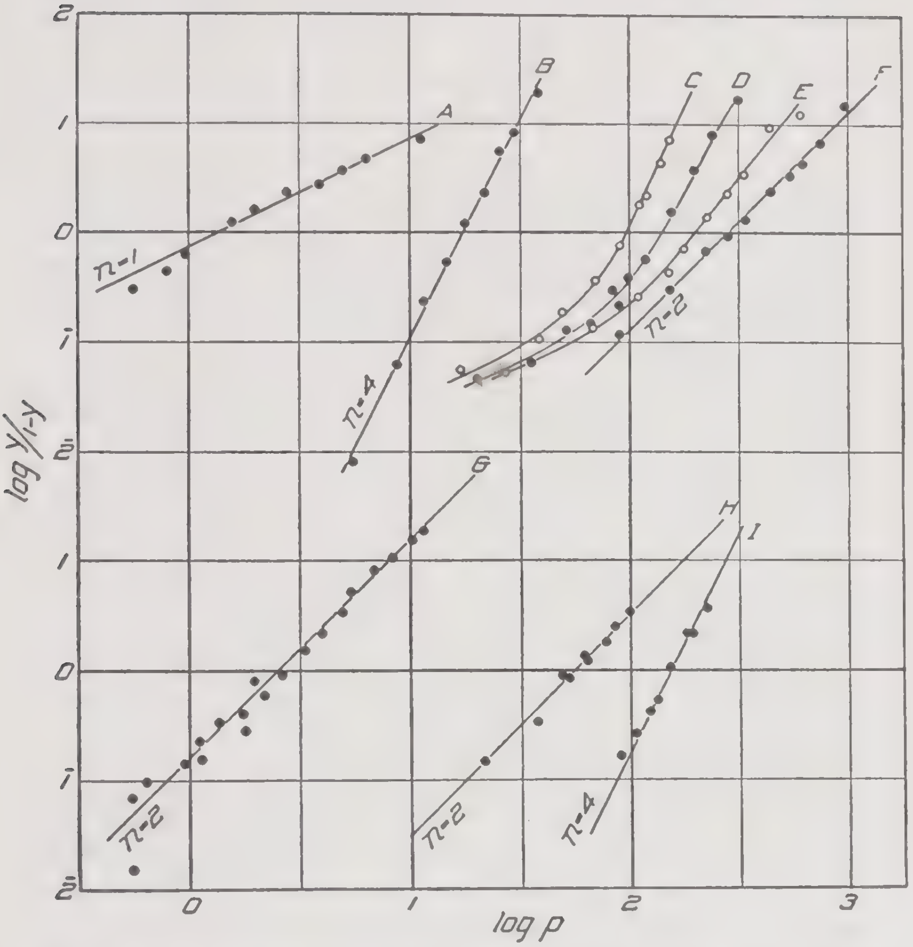

FIG. 2. Oxygen dissociation curves of hemocyanins plotted according to the logarithmic form of Hill's equation. $y/1-y$ is the ratio of oxygenated to reduced hemocyanin, p is the pressure of oxygen. In this plot the points fall along a straight line if n is constant. The slope of the line is determined by the value of n.

A, serum of *Busycon* at pH 5.79 shows $n = 1$.
B, serum of *Homarus* at pH 8.08 shows $n = 4$.
C, D, E, serum of *Homarus* under conditions when n is not a constant and Hill's equation is inadequate.
F, serum of *Homarus* at pH 6.42 shows $n = 2$.
G, H, serum of *Loligo* shows $n = 2$.
I, serum of *Loligo* shows $n = 4$.

From Redfield and Ingalls (28).

systematic way which suggests that the phenomenon of aggregation is closely related to the state of some acidic or basic group having a characteristic dissociation constant (28).

While the successful application of an equation as complex as that required in the present case is not altogether convincing, the phenomena of the oxygen equilibrium make it clear that the prosthetic groups of the hemocyanin molecule may be associated in some way such that they do not each combine with oxygen independently.

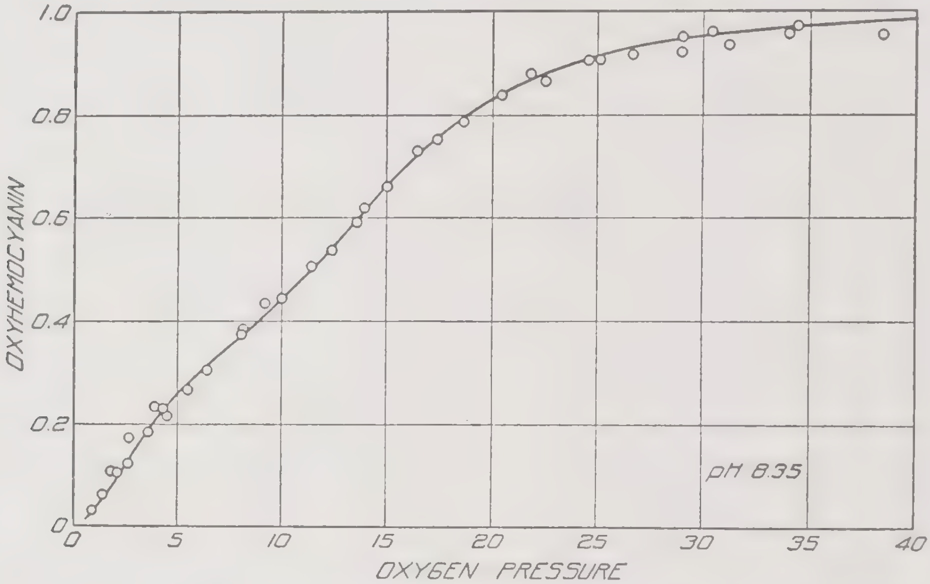

FIG. 3. Oxygen dissociation curve of hemocyanin in blood of *Busycon* at pH 8.35. Curve is calculated assuming two-fifths of prosthetic groups are associated in pairs and three-fifths in groups of four. From Redfield and Ingalls (28).

Pictures of the hemocyanin of *Busycon* made with the electron microscope show the particles to consist of rod-like sub-units which appear to be stacked together in bundles of four (Fig. 4). Usually the rods are grouped with their long axes parallel so as to produce a cube-like composite particle of uniform dimensions. In a few cases, one pair of rods seems to be rotated 90° with respect to the other, so that the tetrad shows the ends of two rods and the side view of another pair. At higher pH, the tendency to form tetrads disappears, and the rods appear singly (18). In the case of this hemocyanin, the association of the rods as groups of four, as crossed pairs, and as single units corresponds approximately, according to the conditions, with the association of the prosthetic groups in ones, twos, and fours as indicated by the oxygen dissociation curves. It would be exciting indeed if it turned out that the electron microscope pictures reveal the structural basis on which the nature of the oxygen equilibrium depends.

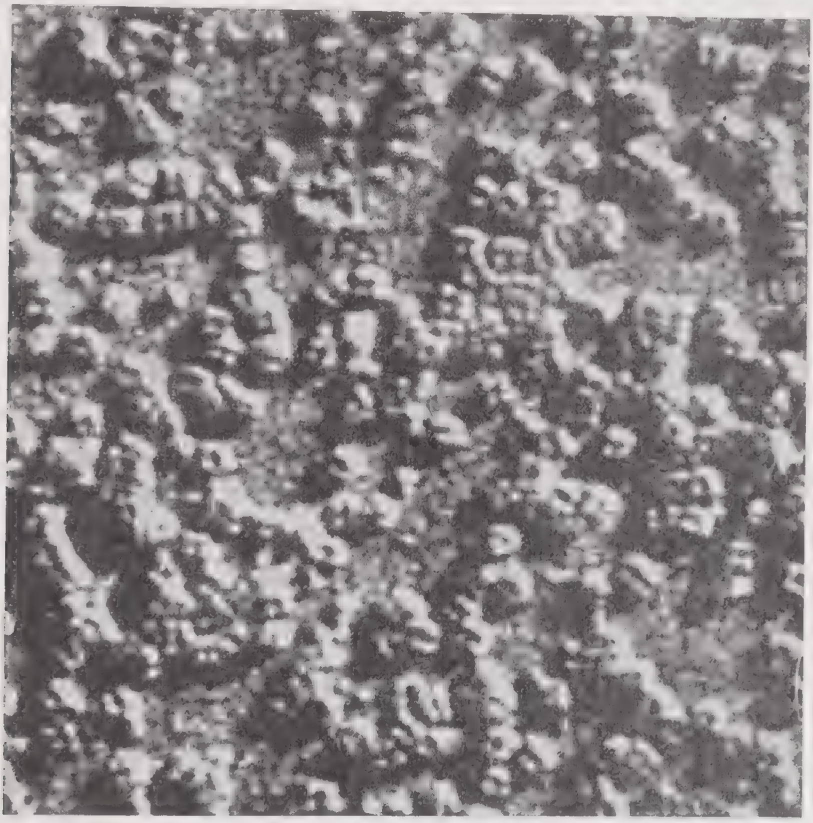

FIG. 4. Electron microscope picture of hemocyanin of *Busycon.* From Polson and Wyckoff (18).

The titration curves of hemocyanins do not differ from those characteristic of proteins in general (26). In *Limulus* hemocyanin some 117 groups capable of neutralizing acid are present in each portion of the molecule having a single prosthetic group. This agrees with the quantity of dibasic amino acids present. The pK value of these groups is 3.3. The base binding groups are equally numerous. While most of the base is bound at reactions in the neighborhood of pH 11, considerable buffer action occurs at more neutral reactions. Although there is no reason to believe that most of the groups involved in neutralizing acid or base have any particular effect on the prosthetic group, there is evidence that some groups at least are closely associated with the part of the molecule which combines with oxygen.

Good evidence for this statement exists in the fact, already alluded to, that changes in the hydrogen ion concentration alter the association of the prosthetic groups and influence their oxygen equilibrium constants. The most precise evidence, however, comes from the reciprocal relation, namely, the effect of oxygenation upon the titration curve of the protein. Shack (31) has shown that oxygenation makes the hemocyanin of the lobster more acidic. This effect may be explained quantitatively on the assumption that the oxygenation of each unit containing one atom of copper decreases the value of the dissociation constant of a single divalent acid- or base-binding group. Somewhat similar relationships appear to exist in the hemocyanin of the crab and the snail, but cannot be detected in *Limulus* hemocyanin. The implication of Shack's study is that in some species each prosthetic group contains, or is closely associated with, two divalent acid- or base-binding groups. In the case of the lobster, the pK value of these groups is changed from about 7.6 to 6.8 upon oxygenation.

Finally, the absorption spectra of the hemocyanins should be mentioned. Reduced hemocyanin appears to absorb light because of the scattering associated with large molecules. When correction is made for this effect, there is found to be no absorption except for a band in the ultraviolet at about 280 mμ, characteristic of proteins in general. When oxygenated, characteristic broad absorption bands appear, with maxima at about 350 and 570 mμ. A careful comparison of the absorption spectra of the hemocyanins of a number of species in the visible range indicates that as a class the hemocyanins have spectra which are similar, but that unquestionable, minor differences distinguish the different species (Fig. 5).

To summarize, the hemocyanin molecule as it exists in the blood is an aggregate of a large number (up to 200) of protein units.

The units of which these giant molecules are composed have molecular weights of 50,000 to 74,000. They are undoubtedly different proteins in different species, though all may be classed as globulins. Each unit carries a single active group, containing two atoms of copper, apparently in the cuprous state. Each of these groups combines reversibly with one molecule of oxygen or carbon monoxide. The active groups may not be altogether independent, two or four being associated in some way in uniting with oxygen. In some hemocyanins, two divalent acid- or base-binding groups are

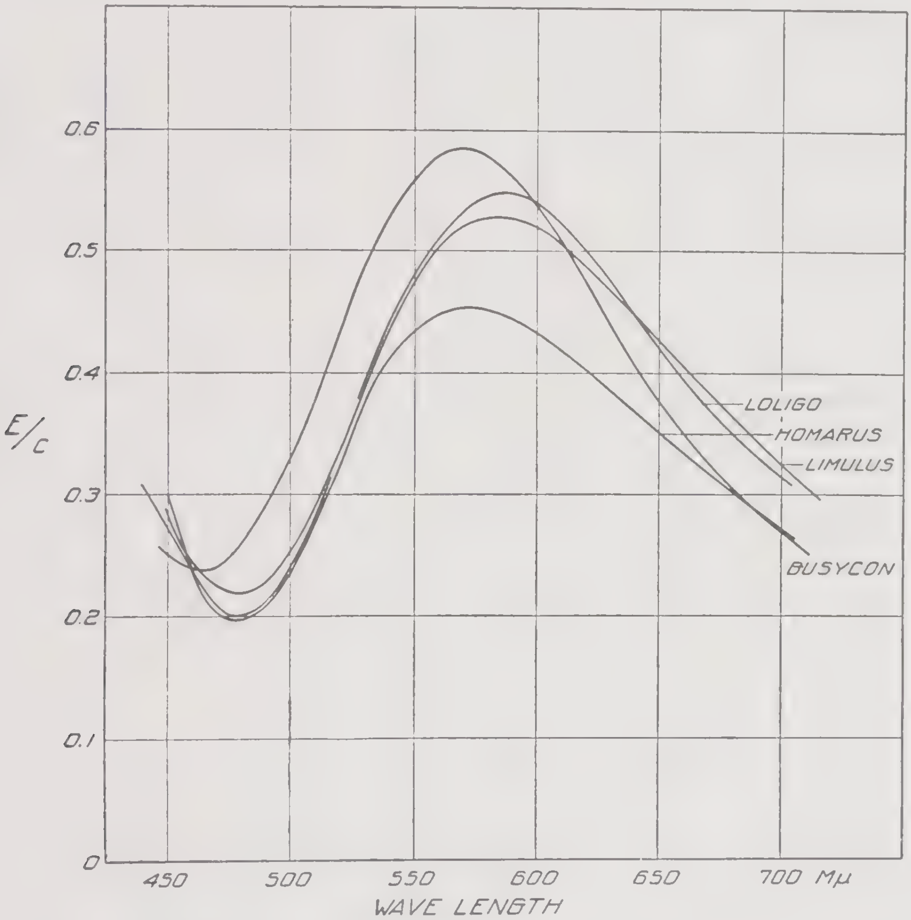

FIG. 5. Absorption spectra of oxyhemocyanin of various species, corrected for the light lost by scattering. E/c, extinction coefficient per milligram atom of copper per liter of solution. From Redfield (23).

closely enough associated with the active group to be influenced by the union with oxygen.

As a class, the hemocyanins are alike in being proteins containing copper, in the combining ratios in which copper combines with oxygen or carbon monoxide, and in the general character of the absorption spectrum of the oxygenated molecule. It should be emphasized that the hemocyanin of each natural group, which has been adequately studied, is a distinct chemical entity. Differences exist in solubility, buffer action, isoelectric point, elementary composition, minimal molecular weight, and degree of molecular aggregation, differences which reflect the specific character of the molecule as a whole.

Differences also exist in the detailed character of the absorption spectrum and in the quantitative relations which control the equilibrium with oxygen. These differences suggest that the active groups of the hemocyanins are not identical. Unfortuately, our knowledge of the chemistry of the prosthetic group is unsatisfactory. Until this is remedied, we cannot understand the relationships among the different hemocyanins or their relation to the other copper proteins.

In discussing the relation of hemoglobin to the development of knowledge of the porphyrin pigments, Anson and Mirsky have written:

> . . . The universal distribution of the porphyrin pigments and the heme nature of the respiratory ferment have been discovered primarily because hemoglobin and its derivatives have been so readily available for study and because these substances have such well defined spectroscopic properties. Once, for example, the reactions and spectra of the hemoglobin derivatives were known, there was no difficulty in identifying cytochrome as a heme compound. . . . (2).

The foregoing summary of our knowledge of hemocyanin has been presented in the belief that this substance may serve similarly as a useful prototype for the understanding of other copper compounds of biological interest.

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DISCUSSION

DR. POULSON: Do you have any idea about the site of synthesis of hemocyanin?

DR. REDFIELD: No, none whatever. There has been one report in *Chemical Abstracts* by an Italian worker, but unfortunately I have not been able to consult the original as yet. Apparently he was able to show that copper appeared in the embryos at certain stages of development prior to the synthesis of hemocyanin.

THE USE OF RADIOISOTOPES OF COPPER AND MOLYBDENUM IN NUTRITIONAL STUDIES

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The essentiality of copper for plants and animals, the association of this element with a definite function in the living organism, and the interrelationships of copper with molybdenum have long been recognized and are discussed elsewhere in this symposium. Convenient and reliable chemical analytical methods for copper and molybdenum, with sensitivities adequate to determine these elements in the amount normally present in animal and plant tissues, have led to a considerable accumulation of data on various aspects of copper metabolism. Nevertheless, further information is needed in regard to the apparently complex function of this element in the living system. The use of radioisotopes in conjunction with other methods offers a dynamic approach which is expected to be of great value.

Some general references on radioisotope techniques include Kamen (9), Hevesy (8), Comar (2), Schweitzer and Whitney (21), University of Wisconsin Symposium (1), Friedlander and Kennedy (6), Lawrence and Hamilton (10), and Siri (23).

Availability of Radioisotopes

Table 1 describes the radioisotopes of copper and molybdenum which are suitable for nutritional studies. The 12.8 hrs. half-life of Cu^{64} limits the period of experimental use to a few days, depending upon the initial activity available, the initial specific activity, the uptake by the tissues of interest, and the sensitivity of measurement. Cyclotron-produced Cu^{64} offers the advantage of having a high specific activity; however, since its cost will be high and the total activities available will be relatively small, this advantage would become of actual benefit only in small scale experimentation. Details in regard to the preparation of target material for use will be described in a later section dealing with specific examples.

Mo^{99} is better suited than copper for animal tracer studies because of its somewhat longer half-life, its relatively low toxicity, and its greater absorption by the animal. The above observations on cyclotron-produced copper would also be valid for cyclotron-produced molybdenum isotopes.

TABLE 1

Radioisotopes of Copper and Molybdenum Suitable for Nutritional Studies

Isotope	Half-life	Radiation Energy (MEV)		Production	Projectile and Target	Form Available	Specific Activity Initially Available mc./g.	Cost ($ per mo.)
		Beta	Gamma					
Cu 64	12.8 hrs.	0.58$\beta-$ 0.66$\beta+$ K	1.2 (weak)	Cyclotron	Protons Ni	....	4000*	....
Cu 64	12.8 hrs.	0.58$\beta-$ 0.66$\beta+$ K	1.2 (weak)	Pile	Neutrons Cu	Cu	300	0.12
Mo 99**	67 hrs.	1.3	0.77, 0.815 0.840	Pile	Neutrons MoO_3	MoO_3	10	0.30

* A preparation reported in (12).

** A preparation containing high specific activity Mo^{93} (6.7 hrs. half-life) and Mo^{99} has been produced by alpha bombardment of ZrO_2 in the cyclotron (24).

Measurement of Radioactivity

The method of measuring the radioactivity will usually be chosen on the basis of the sensitivity required and the ease of preparation of samples. Table 2 presents data on the relative sensitivities of various counting arrangements for copper 64 and molybdenum 99. As expected, the most sensitive measurement would be attained by using a thin film with a mica window counter tube. However, if the samples contain sufficient activity so that a loss of sensitivity from 5 to 100 fold can be sustained, then there is some advantage in making the measurements on the solution because of the greater ease of preparing samples and the reduction in time required for it, a saving which may counteract the loss of sensitivity.

In order to give an approximation of absolute sensitivities it can be stated that the Bale-Haven Dipping Counter with a 2 ml. cell can detect 0.005 micrograms of labeled copper of 70 mc./g. specific activity, and 0.04 micrograms of labeled molybdenum of 5 mc./g. specific activity.

TABLE 2

THE RELATIVE SENSITIVITY OF DIFFERENT COUNTING ARRANGEMENTS FOR COPPER 64 AND MOLYBDENUM 99

Counting Arrangement	Copper 64	Molybdenum 99
Bale-Haven Dipping Counter (background 10 c/m.)		
2 ml. cell	100	100
15 ml. cell	22	18
Eck and Krebs, 30 mg/cm² Glass Wall Counter (background 40 c/cm.)		
Thin film on glass	100	200
2 ml. of solution (petri dish)	4	21
Thin Mica Window Counter (background 40 c/m.)		
Thin film on glass	500	500
2 ml. of solution (petri dish)	12	40

NOTE: Sensitivity for Bale-Haven Dipping Counter with 2 ml. cell set at 100.

PREPARATION OF SAMPLES FOR MEASUREMENT

Consideration is given here only to the preparation of samples for solution measurement, since this has been found most advantageous both for Cu^{64} and Mo^{99}. Other methods will be described in a later section which will review papers published on the use of these isotopes.

The body fluids, such as blood, plasma, urine, bile, or milk, may be assayed directly if sufficient activity is present. If necessary, samples of any of these up to 250 g. can be ashed and concentrated to volumes of about 50 ml. It is desirable to reduce solid samples to a homogeneous liquid. The customary ashing procedures of analytical chemistry may be used; however, it is often possible to shorten them considerably, since the requirements for radioactive assay are not as stringent as for chemical trace analysis.

When the conventional method of dry-ashing in the muffle furnace is used, care must be taken to avoid losses due to volatilization, incorporation of the isotope into solid carbon particles, or adsorption of the element to the crucible. In studies with short-lived Cu^{64}, this method of dry-ashing was found to be too time-consuming, and so a rapid method of pseudo-wet-ashing, based on extraction of the fat by a solvent, was developed so that 200 samples could be prepared for measurement within a few hours (2). In this procedure a representative sample, up to 50 g. in weight, is cut into small pieces and placed in a 400-ml. beaker, to which 40 ml. of concentrated nitric acid are added. The tissue is allowed to soak for 10 minutes, after which gentle heat is applied. The resulting solution is evaporated to about 15 ml. on a hot plate and is then transferred to a steam bath for continued evaporation to remove the acid. The residue is washed into a separatory funnel with small amounts of warm water. The beaker is rinsed with two 10 ml. portions of iso-amyl alcohol, which are in turn transferred to the separatory funnel. The separatory funnel is shaken gently, and the two solutions are separated and made to volume for measurement with a Geiger counter. The activities as measured in each solution are added to give the total for the sample. Iso-amyl alcohol was the most suitable general solvent tried, chiefly because of its high boiling point.

Certain tissues may require slight modifications of this procedure and following are suggestions for such circumstances. When it is necessary to pseudo-ash more than 35 g. of whole blood, it has been found advantageous to slow down the initial reaction by using increased amounts of acid and applying heat very cautiously, with frequent stirring. In the case of samples of tooth, bone, or cartilage, care must be taken not to reduce the acid solution to the point where the salts start to precipitate. It is very difficult to get the precipitated salts back into solution. In handling samples such as white bone marrow, lymph glands, or adipose tissue, which contain large amounts of fat, there is a tendency for bumping and spattering, so that it is advisable to heat them gently at all times, preferably on a water bath. With this type of sample, a solvent mixture of 10 parts of ethyl alcohol, 25 parts of diethyl ether, and 25 parts of petroleum ether may be more efficient than iso-amyl alcohol.

Some feces samples, depending on the nature of the diet, may require more than the usual amount of nitric acid for complete digestion. It has been observed that if these samples are evaporated to dryness, it is very difficult to get the residue into solution again. In some experiments, collections are made on filter paper, and in such cases wet-washing may be carried out as follows: the filter paper is first dampened with water, and 4 to 5 ml. of concentrated sulfuric acid are added. About 10 ml. of nitric acid are then added, the beaker is heated gently until the violent reaction has subsided, and then heated further until the contents are charred. At that stage, 25 to 30 ml. of nitric acid are added, and the digestion continued as previously described.

In some studies, it has been necessary to concentrate samples of milk for the measurements of activity. A convenient procedure, combining the wet- and dry-washing techniques, is as follows: 150 ml. of milk is pipetted into a 400 ml. beaker containing a few glass beads, and 40 ml. of concentrated nitric acid is added. The mixture is heated gently until the foaming has subsided and then evaporated to reduce the volume. The solution is then heated on a steam bath until the excess nitric acid is removed. The solution is transferred to a 90 ml. porcelain crucible, using warm wash water and taking care that the total volume of the sample does not exceed 50 ml. The solution is dried at 100-105°C for about 12 hours and then ashed in a muffle furnace at about 500°C for a minimum of 5 hours. The ash is taken up in about 8 ml. of an acid solution (100 part of 2 N HCl and 20 parts of conc. HNO_3) and made to volume. If it is necessary to concentrate the milk more than 150 to 25, more than one 150 ml. batch can be started, and the final ash solutions combined.

In turnover studies, which require both chemical and radioactivity analyses, the measurements of activity can often be made from aliquots of the analytical solutions, thus requiring very little additional work.

CONSIDERATIONS OF DOSAGE

The dosage of a labeled element administered to an organism must be considered both as to the radioactivity present and as to the total amount of the element involved. The definitions below are concerned with total amount of element administered, it being assumed that there is sufficient radioactivity to make measurements throughout the experiment:

Tracer dose: The amount of the administered element is small compared with the normal intake.

Physiological dose: The amount of the administered element is of the same order of magnitude as the normal intake.

Massive dose: The amount of the administered element far exceeds the normal intake.

Therapeutic dose: Sometimes used when the level of radioactivity in the administered dosage is sufficient to produce a biological response.

It is considered that the tracer dose and physiological dose will follow the normal path of the element in metabolism, since the amount of the element introduced into the system will not be large enough to disturb equilibrium or steady-state conditions. On the other hand, a massive dose may well flood the tissues and bring about an abnormal distribution. The classic example of flooding is the behavior of radioiodine administered to rabbits, as reported by Hertz et al. (7). The percentage collection of the iodine dosage by the thyroid was directly affected by the amount injected; the percentage of collection increased as the dosage was decreased. In terms of a concentration coefficient, which served as a measure of affinity of the thyroid for iodine, the value at a dosage of 0. 1 mg. was about 80, while for an injection of 100 mg. it was found to be 2 or less. In a study of toxicity, however, it would be desirable to use a near-toxic level. This usually presents no problem, since the dosage can be increased merely by using an additional amount of the stable isotope. Difficulties usually arise when it is necessary to keep the dosage at a minimum. An example of a therapeutic dose would be the use of sufficient radioiodine to bring about destruction of thyroid tissue.

The ideal tracer experiment consists in the use of a tracer dose, or if that is not possible, of a physiological dose with a level of activity such that a sample of convenient size gives a count several times that of the counter background at the time of measurement. No radiation effects would be expected at this level of activity except perhaps with short-lived isotopes, where a high initial activity may be required, or in case the accumulation in the particular tissue of interest is very low. In practice it is usually necessary to make a compromise depending largely upon the characteristics of the isotope preparation.

Successful use of Cu^{64} in animal experimentation must take into consideration the toxicity of this element and the short half-life of the isotope. This means that the material used should have a high specific activity and that the preparation of the samples for assay should be as rapid as possible. The toxic level depends largely upon the conditions of administration. For instance, a single dose of 5 mg. of copper as the sulfate injected into the blood stream of a rabbit, or 100 mg. given it in a dilute solution by stomach tube is fatal within a few hours. On the other hand, as much as 200 mg. of copper dried on the food can be administered to a rabbit every day for months with no observable effects. With sheep and swine, 1 to 3 g. doses are used therapeutically, and 4 to 8 g. doses serve for cattle.

RADIOISOTOPE STUDIES WITH COPPER

In 1941, Mazia and Mullins (11) reported the use of Cu^{64} in a study of the oligodynamic action of heavy metal ions. The isotope was prepared in the cyclotron with an activity of about 10 mc. per g. Leaves of *Elodea canadensis* were immersed in 10^{-6}, 10^{-8} and 10^{-10}M solutions of $Cu^{64}Cl_2$, and after a given time of immersion, a known weight of leaf material was washed in 500 volumes of distilled water and analyzed with a Geiger-Müller counter. No further experimental details were described. It was found that the tissues were able to accumulate considerable copper from a low external concentration. The ratio of Cu^{64} inside the leaf to the Cu^{64} outside was in the range of 10^4 to 10^8. It was concluded that copper, in extreme dilutions, exerts its physiological effects by virtue of the power of protoplasm to bind ions against a concentration gradient, so that the concentrations present are relatively high.

Yoshikawa, Hahn, and Bale (25), in 1942, described the use of Cu^{64} to study the uptake of this element by the red blood cells in normal and anemic dogs. Their material was prepared by proton bombardment of nickel, after which the nickel was dissolved in a small amount of concentrated nitric acid, diluted with distilled water, and 2 mg. of inert copper was added as a carrier. After precipitation with H_2S, the copper was dissolved in a few drops of nitric acid and plated onto platinum from a solution of 1 ml. of concentrated HNO_3 and 3 ml. of concentrated H_2SO_4 in 100 ml. of water. The deposited copper was dissolved in concentrated HNO_3 and after drying on a steam bath the residue was made to 25 ml. with water. This solution was mixed with 20 g. dried white bread and 10 g. of salmon for feeding, carrier being added so that 0.8 mg. of copper as the nitrate was fed to each animal.

Samples were prepared for an assay of their radioactivity by ashing with 5 ml. of concentrated H_2SO_4, with a little perchloric acid used as needed. Water was added and, using phenol red as an indicator, neutralization with 40% NaOH was carried out. Then weakly acid (yellow), 20 ml. of saturated $Na_4P_2O_7$ were added, and the solution made just alkaline. After cooling and transferring to a separatory funnel, 30 to 40 ml. of ethyl ether and 2 ml. of 0.1% sodium diethyldithiocarbamate were added, and the ether layer separated after thorough shaking. The water layer was again extracted with ether after a few hundredths of a milligram of copper had been added as carrier. This extraction was repeated three or four times, the extracts were combined and evaporated, a few drops of concentrated HNO_3 were added, and the solution was made to 2 ml. The measurements were made with a dipping-type counter similar to that in the method reported by Comar (2).

Following ingestion of radioactive copper by the dog, the metal appeared quickly in the plasma, the concentration reaching its peak in from 2 to 5 hours, after which it fell abruptly. Demonstrable amounts were still present after 2 days. The radioactive copper likewise appeared rapidly in the circulating red blood cells, and there was a slow but gradual increase in its concentration over a two-day period. An insufficient number of animals was used to permit a definite conclusion, but there was an indication that where the hematopoietic activity was greater than normal, the uptake of the copper by the red blood cells was increased.

In 1942 Schultze and Simmons (20) employed Cu^{64} in studies on nutritional anemia of rats. Nickel targets weighing 200 to 250 mg. were bombarded with 6.7-Mev protons in the cyclotron for 8 to 12 hours; the copper content of the nickel was about 0.1%, so that the sample contained 0.2 to 0.3 mg. of copper. The target was dissolved in a mixture of HCl and HNO_3, and the solution was evaporated, diluted, and repeatedly extracted with a CCl_4 solution of dithizone (0.1 mg. of dithizone per ml. of CCl_4) until the color of the reagent remained unchanged. The CCl_4 extract was evaporated to dryness, ashed with 0.2 ml. of concentrated H_2SO_4 and a few drops of H_2O_2, and then diluted with 0.8 ml. of water. The acid was neutralized to phenol red with redistilled ammonia and made to about 2.5 ml. The activity of these samples at the time of feeding was about 0.5 millicuries. Of this solution, 1 ml. was fed to each rat and 0.1 ml. was used as a calibration standard and for chemical determination of total copper. The radioactive material was fed to the rats in a small amount of milk; since the animals were fasted for 14 to 16 hours before feeding, rapid consumption resulted; however, any residue left in the feed dish was determined by analysis and corrections applied.

Except for the carcass and different parts of the intestinal tract, the samples were prepared for measurement as follows: the tissues were weighed, transferred to glass-stoppered digestion flasks of about 60 ml. capacity, and ashed with sulfuric and perchloric acids. After complete digestion, the acid solution was diluted and neutralized to about pH 2, with patent blue V as indicator. To this solution 3 ml. of dithizone in CCl_4 were added, and the copper was extracted by mechanical shaking for 15 minutes. A solution of dithizone containing 0.1 mg. per ml. CCl_4 was used. The CCl_4 layer was used directly for radioactivity measurement. Under these conditions, quantitative recovery was obtained, either with or without added tissue. Since the feces and intestinal tract contained considerable activity, they were wet-ashed, and the ash solutions made to volume and measured directly; this was also done with the carcasses, except that they were dry-ashed. Measurements were made with the dipping-type counter, as described in (2). In aqueous solutions no corrections were necessary for self-absorption by the solvent; however, with CCl_4 solutions, only 85 per cent of the corresponding activity in aqueous solution was registered in the counter, so that a correction was applied.

TABLE 3

The Distribution of Copper Fed to Anemic Rats (20)
Copper Dosage 0.1 to 0.15 mg. per rat

Type of deficiency— Duration of therapy— Number of rats—	Copper 24 hrs. 9	Iron 24 hrs. 4
	Per cent of Copper Dosage	
Blood	0.54	0.16
Liver	1.57	0.40
Kidneys	0.56	0.32
Spleen	0.14	0.02
Bone marrow	0.05	0.01
Carcass	2.54	1.43
Gastro-intestinal tract plus excreta	70.00	74.93
Retained	5.40	2.34
Recovered	75.40	77.27

Typical results are presented in Table 3. The rather low retention of copper by the animal is well illustrated. There was no explanation for the low total recovery of the administered copper. The iron-deficient rats, which were receiving adequate copper, showed a somewhat lower retention than the copper-deficient group. Of the radiocopper in the whole blood, from 49 and 75 per cent was contributed by the plasma. It was very interesting that per unit weight the kidneys showed $1\frac{1}{2}$ to 3 times more radioactive copper than the liver. It was calculated that the deposition of copper in the bone marrow was about the same order of magnitude as in the liver, and was sufficient to elicit an increase in cytochrome C oxidase activity and to initiate hematopoietic activity. This demonstrated the entrance of therapeutic copper into the hematopoietic centers in amounts that were too small to permit detection by chemical methods.

In 1943, a group of German workers (13) reported the use of Cu^{64} for injection studies, using dogs as subjects. The isotope was prepared by bombardment of metallic copper with deuterons. The irradiated target was dissolved in concentrated H_2SO_4 and used as copper sulfate. About four or five hours after irradiation, the radioactive copper sulfate in physiological salt solution was intravenously injected into two dogs. With one animal, the total activity after 15 hours was 870 μc. in 39.5 mg. of $CuSO_4$; with the other, it was 523 μc in 62 mg. $CuSO_4$ after 25 hours. The samples were treated with H_2SO_4 and fuming HNO_3, and heated to complete oxidation. After neutralizing with concentrated ammonia and the addition of about 10 mg. of inert copper, the metal was precipitated in weak acid solution with sodium sulfide. The solution was filtered and, with samples of high activity, the measurements on the filter paper were made with an ionization chamber. The activities of weak preparations were measured with a Geiger-Müller counter.

It was observed that the Cu^{64} was rapidly removed from the blood, a fact indicating that the tissues picked up a considerable amount of the element from the first capillary passage. The copper in the red blood cells increased slowly; however, it remained at a high level for a longer period of time than in the plasma. The liver showed the largest copper accumulation of all the organs and also the greatest amount of copper per unit weight. Secretion of the copper in the bile occurred relatively slowly, and it was suggested that the high copper of the intestinal contents was derived in part from secretion by the mucous membranes of the small and large intestine. It was interesting that of the radioactive copper in the kidney, most was found in the cortex. Relatively small amounts were found in the muscle tissue and even smaller amounts in the nerve tissues.

The use of pile-produced Cu^{64} in studies of large as well as small animals has been reported by Comar, Davis, and Singer (3). It was considered desirable to place emphasis on experimentation with cattle, in view of the apparent increased sensitivity of the ruminant species to copper deficiency.

The irradiation unit consisted of 0.32 g. of pure copper wire. As removed from the pile, it had about 100 mc. activity. At the time of use the unit had decayed to about 20 mc. The copper was removed from the shipping container, immediately dissolved in a small volume of 8 N nitric acid, and was then neutralized with 2 N sodium hydroxide. A magnetic stirrer was used to provide vigorous agitation during neutralization in order to avoid precipitation of the hydroxide; the solution was adjusted as close as possible to neutrality without causing precipitation. Measured amounts were then removed for calibration standards and administration to the animals. Health precautions, handling of the samples, and procedures for radioactive assay have been discussed in detail by Comar (2).

From theoretical considerations, no significant amounts of radiocontaminants would be anticipated in the unit, and this would lead to the expectation that there would be no practical advantage gained by purifying the copper as received. The half-life of each sample was determined and found to be in good agreement with the established value of 12.8 hrs. When the copper was purified by extraction from acid solution with dithizone, there was no observable change in its half-life. Perhaps of most significance were experiments in which the blood plasma was analyzed chemically for copper before and after administration of the radioactive preparation. The increase in plasma copper as determined chemically agreed fairly well with the values obtained by activity measurements. This means that in so far as accumulation in the plasma is concerned the stable and radioactive atoms of copper behave identically.

The actual dosages used depended upon the specific activity of the preparation, the accumulation expected in the tissues, and the

anticipated duration of the experiment. With rats, about 1 to 8 mg. of copper were used, with rabbits from 7 to 25 mg., and with cattle from 100 to 250 mg. of copper.

It was apparent from excretion studies with all species that the absorption even of soluble copper was quite low. During the five days after administration, about 75 per cent of the soluble copper fed to cattle was excreted in the feces and about 3 per cent in the urine. On the other hand, when the copper was injected intravenously, only 6 per cent of the dose was excreted in a like period, about equally in the feces and urine. This apparently high retention of injected copper confirmed a field experiment with cattle, which had demonstrated that injected copper exhibits a much longer period of therapeutic efficiency than orally administered copper.

Table 4 presents typical values of the tissue distribution of labeled copper administered to cattle. It is evident that although the retention by the animal is low, the copper which was absorbed is generally distributed and reaches the tissues involved in hematopoiesis. The tissues showing a high concentration were, in decreasing order, the liver, kidney, gastro-intestinal tract, adrenals, thymus, gall bladder, and bile. Those of medium concentration included the pancreas, red bone marrow, intestinal lymph, blood, spleen, heart, lung, and reproductive organs. Very low concentrations of copper were found in the white bone marrow, muscle, hide, bladder, ligament, cartilage, bone, eye, and nerve tissue.

It is obvious that the liver, which showed the highest concentration, serves as the chief storage organ. The kidney had the next to highest concentration and in adult cattle the ratio of liver to kidney accumulation averaged about five. In tissue distribution studies with rats sacrificed after 17 to 65 hrs., the concentration of labeled copper in the kidney was found to exceed that in the liver, which points to a species difference and confirms the results of Schultze and Simmons (20). With rabbits, the liver-kidney ratio of labeled copper ranged from 13 to 20. Chemical analysis has shown that the livers of very young animals are especially high in copper, and that a calf is born with a copper concentration in the liver of 4 to 8 times that in the liver of the adult. The data in

Table 4 confirm this selectivity and indicate that it persists at least through the first 1 ½ months of life. In comparing the values from young and adult animals, it is better to use the percentages of dose, since the concentrations are affected by body weight. It is to be noted that, except for the liver, the percentages of dose found in the various tissues are in close agreement. This indicates that the liver of the calf actually exhibits a greater selective accumulation of the absorbed copper than the other tissues.

In general, tissue values following injection were about 10 times the corresponding values after ingestion, a ratio which again reflects the poor absorption of orally administered copper. Proportional distribution of the injected copper was similar to that of the ingested copper.

Since it is recognized that the gastrointestinal tract has an important hematopoietic function, particular attention was paid to the accumulation of copper in the true stomach and intestinal mucosa. It may be noted from Table 4 that in all cases the mucosal was greater than the muscular concentration, negative values for the duodenum being due in part to the small sample available. The ratio of mucosal to muscular concentration averaged 2.7 for ingested and 2.8 for injected copper. Where the copper was injected, the accumulation in the mucosa probably occurred via the general circulation, since it has been shown that relatively small amounts of copper reach the intestinal contents during the 24 hours following injection. Particularly in the case of the true stomach would it seem unlikely that the mucosal accumulation of the injected copper could have come from the stomach contents. It is considered, then, that the gastrointestinal mucosa does concentrate copper from the general circulation, but an estimate of the extent of secretion into the lumen must await bile and pancreatic fistula studies.

Blood studies in cattle with Cu^{64} have led to the following picture in regard to the behavior of intravenously injected copper. Immediately after injection two processes occur, removal of the copper from the plasma by deposition in the tissues, and absorption of copper by the red blood cells. The first process occurs initially at a much faster rate than the second. After the large initial absorption of copper by the tissues, which takes place within five minutes after injection, there is practically no removal of copper from the blood for about an hour, but the red blood cells continue to accumulate copper from the plasma. Between one and three hours after injection there is further removal of copper from the blood, an indication that most, if not all, of the copper is being removed by the tissues from the plasma. From then on, attainment of equilibrium between the red blood cell and plasma copper proceeds at a faster rate than the removal of copper from the blood.

The low copper content of milk has long been recognized. In order to study directly the secretion of dietary copper in the milk, 289 mg. of labeled copper were fed to a dairy cow. Only 0.005 per cent of the dose was found in the milk (5).

Smith and Gray (22) have reported on the distribution of Cu^{64} in early embryo chicks. Radioactive Cu^{64} was converted to the chloride, and the solution was diluted with 0.9 per cent saline solution to contain 150 μc. Cu^{64} per ml. White Leghorn eggs each received, by injection into the albumen, 150 μc. Cu^{64} at 0, 24, and 48 hrs. of incubation. Autoradiographs, which proved more reliable than the Geiger-Müller counter in these experiments, were obtained of the injected embryos between 15 and 84 hours of incubation. Dirctions were given for removing, fixing and mounting the embryos, and for obtaining the autoradiographs on Eastman No-Screen x-ray safety film. Since the relative amount of Cu^{64} entering each embryo depends upon the amount of copper normally present, the proper exposure for each autoradiograph required great care. These pictures showed that copper tends to be distributed in certain patterns: (1) a primary anteroposterior gradient, from the 4-somite stage to the 78-hour stage, starts in the head-fold stage, becomes very noticeable, and tends to vanish after 78 hours; (2) a secondary posterior center starts at the 22 somite stage and is probably associated with the development of the tail; (3) a slight mediolateral gradient appears at 43 to 46 hours; (4) a striking concentration appears in rapidly developing and (or) growing structures, generally beginning with their development or shortly after. These patterns of copper concentration closely paralleled the patterns of metabolic rate, cytochrome oxidase activity, and oxygen reported by other workers. It was concluded that copper distribution in the early chick embryo may be associated with the cytochrome system.

Schubert and Riezler (15) in 1947 reported the use of radioactive copper with human subjects to investigate the absorption of parenterally and enterally supplied doses of copper, and also to investigate the circulation time. All human subjects received intravenously 225 μc. in 0.71 mg. copper into the right elbow vein, 4 subjects received subcutaneously 75 μc. in 1.22 mg. of copper, and 17 subjects received orally 380 μc. in 3.79 mg. of copper. The left elbow vein was punctured simultaneously to take the blood samples. After intravenous injections, qualitative tests showed that activity appeared in 5 seconds. Quantitative tests showed that the maximum activity was reached between 50 and 75 seconds, but it was never more than 60 per cent of the original activity. After 30 minutes, 7.2 per cent of the activity was found and, after 2 hours, 3.9 per cent. Small traces of activity were still detected after 5 days. Following subcutaneous injections, activity was detected in 25 seconds, the maximum activity was reached at 10 to 15 minutes, but not more than 2 per cent of the original activity was found. The orally treated subjects showed maximum activity in twelve hours, but this amounted to less than two per cent of the original. Determination of the distribution of radioactive copper between plasma and blood cells showed that an equal distribution was eventually obtained. Only after subcutaneous injections did the blood cells have less activity. The ionic exchange between plasma cells was found to proceed very slowly.

Continuing this work, Schubert and coworkers (16) reported in 1948 investigations of the absorption, storage, and the excretion of radioactive copper in animals and man. Cu^{64}, with a specific activity of about 0.5 μc./mg., was administered to rats, guinea pigs, dogs, and men, subcutaneously, intravenously, or orally. The distribution pattern of the copper varied between species, and within any one species was affected by the route of administration and the time after administration. The liver, kidney, spleen, bone marrow, and colon showed the highest specific ratios. Only traces appeared in the urine, and the feces were the chief route of excretion, while the bile contributed a minor portion. After oral administration to dogs, 80 per cent was found in the feces in the first 3 days, whereas after a single intravenous dose about 20 per cent was excreted during the first five days with the kidneys excreting a larger fraction than they did after oral administration. Differences in the rate of fixation and specific distribution were interpreted as evidences of active regulation of copper metabolism.

Schubert, Maurer, and Riesler (17) have also used radioactive copper to study the effect of pretreatment with copper or iron. Rats were given, as a single dose, small (5 μc.) or large (250 μc.) amounts of labeled copper as the sulfate. Guinea pigs were pretreated in three ways before receiving one subcutaneous test dose of 25 μc. of Cu^{64}: (a) 25 μg. of copper injected subcutaneously on 13 successive days; (b) 50 μg. of iron injected subcutaneously on 3 or 4 successive days; (c) 500 μg. of copper injected subcutaneously on each of 3 days, or 250 μg. on each of 6 days. In an additional study 1.25 mg. of copper was injected into guinea pigs 12 hours after the test dose alone. The results showed that within this physiological range the size of the dose influences the pattern of distribution. The liver responded as a storage organ with ability to regulate the copper blood level. Pretreatment with iron or copper did not affect the Cu^{64} distribution in the plasma, liver, or spleen while raising it in the red cells, kidneys, and lungs. With high copper pretreatment, the liver took up greater amounts of the Cu^{64}. Injection of inactive copper after the test dose gave lower levels of Cu^{64} in the spleen, kidneys, and lungs, while the Cu^{64} in the liver and bone marrow was further increased.

The same workers (18) have also reported that the radiocopper capacity of the blood plasma is only half as high in anemic rats as in controls. This condition was interpreted in terms of the bindability of the endogenous copper ions on the basis of the catalytic activity of the blood. These workers have also reported on the effect of pregnancy on radiocopper uptake and transfer to the fetus (19). The copper content of the livers of pregnant guinea pigs was found to be higher than that of normal controls. In further investigations, the pathological effects following injection into dogs of labeled $CuSO_4$, to give a radiation intensity of 0.1 to 0.4 millicuries per kg. body weight, were studied (14). Twelve hours after injection leucocytosis, a decrease in lymphocytes, and an increase in neutrophilic granulocytes and eosinophils were observed.

RADIOISOTOPE STUDIES WITH MOLYBDENUM

In 1949, Comar, Singer, and Davis (4) reported the use of Mo^{99} in nutritional studies. Pile-produced Mo^{99} was obtained in 10 g. of MoO_3. The material as received was transferred to a 400-ml. beaker and 35 ml. of 14.3% NaOH was added. The mixture was stirred mechanically and a clear solution obtained in 1 to 12 hours. The solution was then made to a convenient volume with distilled water for use. The pH was near 7. No significant radiocontaminants would be expected in this unit on the basis of the analysis of the target material; the measured half-life agreed well with the established value of 67 hours.

When Mo^{99} was fed to a steer, about 34 per cent of the dose appeared in the feces during 14 days and about 45 per cent in the urine. This indicated the relatively high absorption of soluble molybdenum. Following intravenous injection, about 11 per cent of the dose appeared in the feces and 37 per cent in the urine after six days. Complete tissue distributions have been determined similar to those reported in Table 4 for copper. Highest concentration of labeled molybdenum were found in the bone, liver, teeth, intestinal lymph glands, intestinal mucosa, kidneys, spleen, eye, and red bone marrow; low values were found for the brain, and intermediate values for other tissues. Table 5 shows the accumulation of ingested Mo^{99} in the blood and liver of a heifer in percentages of the administered dose. The total blood was calculated as 7.7 per cent of the body weight. The liver samples were taken by biopsy and the total weight of the liver estimated from experience. The peak of accumulation was reached in both blood and liver at about 41 hours, with the concentrations decreasing thereafter.

Through the use of Cu^{64}, Mo^{99}, and P^{32}, the following observations have been made on rats:

TABLE 5

ACCUMULATION OF INGESTED Mo^{99} IN BLOOD AND LIVER OF CATTLE (2, 4)
(Per cent of Administered Dose)

Time (Hrs.)	Blood	Liver
16	0.60	0.26
41	2.9	0.51
64	2.6	0.42
95	1.5	....
114	....	0.35
138	0.70	....
186	0.19	....
306	0.07	....

(1) Simultaneous administration of copper with phosphorus has little effect on the accumulation of the phosphorus.

(2) Simultaneous administration of molybdenum increases phosphorus uptake.

(3) Simultaneous administration of copper and molybdenum tends to reduce phosphorus uptake.

(4) When molybdenum is fed previously, the phosphorus accumulation is decreased.

(5) Simultaneous administration of phosphorus and/or molybdenum with copper decreases the accumulation of copper in the liver.

In view of the similarity in the metabolic behavior of molybdenum and phosphorus in the animal, an experiment was performed to determine the extent to which dietary molybdenum is secreted in milk (5). About 7 g. of the Mo^{99} preparation was fed to a dairy cow, and the milk collected for radioactive assay. About 2 per cent of the dose given was found in the milk over a five-day period. This was somewhat lower than phosphorus secretion, of which about 10 per cent is usually recovered; but it is much higher than copper secretion.

Nielands, Strong, and Elvehjem (12) have recently reported on molybdenum in the nutrition of the rat. It was noted that with growing rats, a level of 500 mg. per cent of molybdenum as sodium molybdate resulted in death after the first week, whereas levels of 100 and 50 mg. per cent permitted a greatly reduced growth rate. These rats showed no obvious gross pathology or change in blood picture. The toxicity of a diet containing 40 mg. per cent of molybdenum was largely overcome by feeding an additional 2 mg. per cent of copper to a diet already containing 7.73 mg. per cent of copper. Whole liver substance protected against molybdenum, but neither iron, zinc, nor cobalt showed such an effect.

The same workers also used pile-produced Mo^{99} in tissue distribution studies. The sample of radioactive molybdic acid was dissolved in a stoichiometric amount of concentrated sodium hydroxide, and the neutral solution was diluted to 50 ml. of stock solution. The rat tissues were weighed in tared porcelain crucibles and ashed in a muffle furnace after caramelizing at 100°C. The ash was weighed, dissolved, or suspended as evenly as possible in concentrated hydrochloric acid, and an aliquot was transferred to a 50 mm. lead dish and dried for Geiger counter assay.

The general tissue distribution was similar to that already described for this element. The simultaneous administration of a dose of copper did not influence the distribution of the molybdenum. However, all tissues from two rats fed a simultaneous dose of catechol contained considerably more molybdenum after one day than the control group, which received molybdenum only. It would appear that the therapeutic effect of copper is not a simple formation of a less toxic, insoluble copper molybdate.

Stout and Meagher (24) have reported the use in plant studies of Mo^{93} and Mo^{99} of high specific activity, produced through the reactions $Zr^{90}(\alpha n)Mo^{93}$ and $Zr^{96}(\alpha n)Mo^{99}$. The absorption and subsequent distribution of a single microgram of molybdenum in tomato plants grown in complete culture solutions was determined and also the effects thereon of various inorganic ions. The roots accumulated molybdenum rapidly, but translocation to upper parts of the plant was strongly influenced by the phosphate ion concentration in the culture solution. Radioautographs showed that the characteristic patterns of accumulation were distinctly different from those of other mineral nutrients such as potassium, phosphorus, manganese, and zinc. Accumulation of molybdenum occurred around points of greatest water loss in the leaves and appeared to be involved in the reduction of nitrates in the metabolic cycle.

SUMMARY

Little more than a good beginning has been made in the application of radioisotope techniques to problems of minor element nutrition. An overall picture has been obtained of the behavior of copper in the animal body. The high points are the poor absorption of ingested copper, the entrance of copper into the hematopoietic centers, the liver storage of this element, the nature of the equilibrium between red blood cell and plasma copper, the mucosal accumulation of copper, and the increased liver storage of copper by very young individuals.

Through the use of radioisotopes of copper, molybdenum, and phosphorus the physiological interrelationships of these elements have been studied; certain possible mechanisms have been ruled out and others are suggested.

It is anticipated that widespread use of these methods, in conjunction with conventional procedures, will result from the increased availability of isotopes, trained personnel, adequate instrumentation, and the development of specific techniques.

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Discussion

Dr. Talbot: I understand that it is possible to obtain a ten-fold increase in sensitivity by using the activation method that you mentioned?

Dr. Comar: This would depend entirely upon the particular elements. The important points are the cross-section of the element for neutron capture and also the neutron flux to which it can be exposed; we would have to put that on a specific element basis to give you the answer.

Dr. STEINBERG: At the present time, then, can we get within the range of one part per billion?

Dr. COMAR: It is quite possible, but unfortunately the high flux piles are not too available in this country. There is one in Canada, which is much better than the one we have at Oak Ridge. But certainly I feel that there are possibilities in this method which warrant additional work.

Dr. VALLEE: Dr. Austin Brues of the Argonne Laboratory has had some experience with activation analyses for this purpose, and he is very much discouraged. Some work from the Berkeley laboratory also published in *Science* was not impressive. The only results reported were obtained with toxic doses of gold in animals. There is a question I would like to ask: It seems that the results have been expressed in per cent of radioactivity, and no data were given on the actual total amount of copper or molybdenum in the tissues as determined by chemical means. We found in some of our work on zinc that the total zinc measurements and data obtained by radioactivity measurements do not really run parallel. The radioactive data express the metabolic turnover of tissue under examination while the total zinc measurements seem to represent both metabolic activity and inactivity. We found in the stomach that the total amount of zinc was very low indeed, but there was very high accumulation of radioactive zinc, indicating a very rapid turnover.

Dr. COMAR: Let me comment on your first remark: It is true that some of the first work on activation analysis has not been encouraging. However, it would be a shame if they are not exploited further, as there may be great possibilities which will become apparent when more experience has been gained. It's true that turnover data are very desirable and chemical analysis should be run whenever possible. In the cobalt work it was almost impossible, or at least impracticable, to run cobalt analyses on these various tissues. It would have meant that we would have had to cut the amount of work we could have done by a factor of 100 if we had attempted to run cobalt determination. The same is true, but not to quite the same extent, with copper; everyone will agree that having the chemical analysis so that you can calculate turnover data is very important and is very desirable when it is practical. We are doing the same thing now with calcium, and we are going to great efforts to get the analytical data. We feel that it is very worthwhile, and we can make further interpretations which we could not make with the straight activity data alone.

Dr. C. R. DAWSON: Does varying the dose give you information on the rate of turnover?

Dr. COMAR: What we try to do is to adjust the dose so that the action will be independent of the dose. In other words, if we can keep the dose low enough, we feel that this would be true. For instance, in the cobalt work if we would give to a 1,000-pound steer some 50 μg. of cobalt, it would behave in the same way as if had given 200 μg. of cobalt. This is all involved in your dosage consideration. It is ideal to try to keep the dose in line with what you want to find out. In a nutritional study the dose should be equal to or lower than the physiological level. If you were interested in studying the toxic action of copper, then you would be justified in going to a dose that would be near the toxic level, and this would behave somewhat differently from the physiological dose.

Dr. C. R. DAWSON: You don't have any evidence that by varying your dose by a factor of 2 you would alter the analysis?

Dr. TALBOT: I think you are confusing size with magnitude. You are saying that there is a level at which size of dose does not matter, below a certain point. Is that right? In other words, if you get below a certain dose it doesn't make any difference?

Dr. DAVIS: Dr. Comar, you would say in our copper work the limitation was the amount of copper you could give the animal?

Dr. COMAR: That is very true. We were perhaps getting toward the upper limit on the copper work simply by virtue of the specific activity.

Dr. DAVIS: We ran into a problem where we were administering Ca^{45} and Mo^{99} simultaneously and trying to calculate how much radiation the animal could stand, that is, how much could we give them and still find some molybdenum later on. We have to take into consideration that there is a limit somewhere along the line.

Dr. STEINBERG: Have you any evidence, Dr. Comar, as to which compounds these particular elements enter?

Dr. COMAR: No. We have not investigated any of these problems. The excreted cobalt which we analyzed was probably in an organic complex, but we have not followed up this particular line of inevstigation.

Dr. ARNON: Would Dr. Comar care to comment on the interrelationship of phosphorus and molybdenum in the animal, inasmuch as an interrelationship in the plant has also been observed?

Dr. COMAR: Data other than what I have presented indicate molybdenum behaves very similarly to phosphorus in regard to its accumulation in the body; there isn't much more, however, to say on that point. However, I could say that in almost all respects they behave similarly; for example, both are excreted to the same extent in milk.

Dr. ARNON: What I had in mind particularly was not whether there was a parallel distribution, but whether you suspect some metabolic or physiological relationship.

Dr. COMAR: I can't hazard a comment on that.

Dr. BEAR: Do you know whether molybdenum and copper are secreted into the milk in the same ratio as they go into the feed?

Dr. COMAR: I would say that the secretion of copper into the milk is very low, even compared to the dietary intake.

Dr. BEAR: Does the ratio vary widely from the feed to the milk?

Dr. COMAR: I would say yes, very much. I would have to do a little checking on this point, but I am sure we have the data which would give you an answer.

Dr. DAVIS: I can give you some of that information, Dr. Bear. We have been concerned with it. You can triple, quadruple, and even multiply by ten the amount of copper you are giving an animal, and you will not boost the copper content of the milk by more than a factor of about one. You can just about double it in so far as our study is concerned. In the case of molybdenum the situation is entirely different. Even doubling the amount of molybdenum in the ration will greatly increase the amount of molybdenum in the milk, particularly if you are at a low dietary level of molybdenum. If you put one to two parts of molybdenum in the feed, you will increase the molybdenum content in the milk by a factor of ten. Normally milk has practically no molybdenum.

Dr. STEINBERG: Dr. Davis, didn't the Wisconsin group do quite a bit of work on the copper content of milk? In fact, they were able to induce anemia in pigs by feeding them on a strict milk diet.

Dr. DAVIS: That's the ideal ration for producing anemia in rats or pigs. They have also found that it is difficult to raise the copper content of milk by feeding copper.

Dr. THOMPSON: To what extent will molybdenum inhibit phosphatase?

Dr. DAVIS: I can't answer that outright. Molybdenum in the diet tends to increase the alkaline blood phosphatase, whether indirectly, as I suspect, or directly I am not sure.

Dr. COMAR: It has been shown very definitely that molybdenum will inhibit certain plant phosphatases, although I cannot recall the levels involved.

THE INFLUENCE OF COPPER ON THE METABOLISM OF PHOSPHORUS AND MOLYBDENUM

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The apparent influence of copper on the normal formation of bone in cattle under practical animal husbandry conditions, in areas where phosphorus is apparently present at normal levels in the feed, is very striking. In certain areas of this country and others, the animals are unable to form bone properly without copper supplements, with the result that a condition very similar to rickets occurs in young calves, while in older animals there develops a condition of osteomalacia. The abnormality in bone formation is evidenced by broken bones and enlarged joints, but other evidences of malnutrition are also apparent.

Included in our observations have been low hemoglobin values, often as low as 5 grams of hemoglobin per 100 ml. of blood, emaciation, achromotrichia, a stiffening in the joints, an erosion of the joints, and occasionally an abnormal gait, that resembled the pacing gait of a horse and has been called " paces." The texture of the haircoat was rough, sores which developed in the skin failed to heal normally, and death was to be expected unless the animals were moved to a different, unaffected pasture or given some supplemental feed, such as cottonseed meal from a different area.

In the course of experimental work, it was discovered that copper, applied either as a spray or in the fertilizer, had a beneficial effect upon plant growth. With this background, it was natural to attempt copper therapy and prophylaxis with cattle grazing on these areas. Copper sulfate administrations in large measure corrected the difficulties that were being experienced, and served as a base for experimental work on the influence of copper on the metabolism of cattle. When molybdenum was found to be present in these areas, the influence of copper on molybdenum and vice versa was a logical expansion of these studies.

In order to obtain reliable results, it was found necessary to develop a virgin area upon which there had been no experimental fertilizer or truck garden experiments. A 10-area was selected and established in St. Augustine grass (*Stenatophorum secundatum* (Walt.) Kuntze). The St. Augustine grass was used because of its low copper requirement, making it possible to establish a pasture without copper fertilization.

In order to have a water supply that was free from fertilizer contamination, a well was placed in the center of this virgin pasture, and water was supplied to the animals by a watering trough which was open to each of four pastures centering at this point. At the University of Florida Everglades Experiment Station it was possible to establish pastures rapidly, and within three months after the first planting of vegetative cuttings of St. Augustine grass, the cattle were turned into the pasture to use it as their sole source of feed.

An analysis of the grass, as it was prepared and ready for the cattle, is of interest in view of our concern with copper, molybdenum, and phosphorus, and their influence upon each other in the nutrition of the cattle. The phosphorus level of the grass varied from 0.17 to 0.22 per cent, on the basis of dry matter. This is well above the minimum that has been established for cattle in other areas, a level which is usually stated as above 0.13 per cent of phosphorus in the grass. The copper level, as found by analysis of the plants of these pastures, both weeds and St. Augustine grass, ranged from a maximum of 4.2 parts per million to a minimum of 2.8 parts per million. The native weeds were somewhat lower in copper, ranging from 2 to 3 parts per million on the average. Molybdenum values for these same samples ranged from 1.1 parts per million to a high of 1.9 parts per million. The water was likewise analyzed for molybdenum, after symptoms had developed in the cattle, and was found to range from 2.8 to 4.2 micrograms per 100 ml., over a period of 6 months.

These molybdenum values in grass and water were low in comparison with forage and some of the water adjacent areas of peat soil. In areas where similar abnormalities of bone formation were developing, molybdenum was found to range up to 26 parts per million in the forage, with an average of between 11 and 16 parts per million. The water has ranged from 8.5 micrograms per 100 ml. for some well water to 0.0 micrograms per 100 ml. for some surface ponds.

Under our experimental conditions, the two calves which were placed on the pasture with the other animals showed the changes described more rapidly than the older animals. Within 60 days, the ends of their long bones showed swelling, there was beading of the ribs, and a typical rachitic picture appeared to be developing. After 3 months, the first of these calves was put on a supplement of copper amounting to three g. of copper sulfate given as a drench in water once every 10 days. Within a period of 30 days they had begun to show rapid improvement. The dam was also treated with one g. per 100 lbs. of weight at 10-day intervals and made a similar improvement. The second calf was carried on the pasture without supplementation for an additional 60 days. At that time it suffered simultaneous fractures of both scapulae. The vertebral column appeared to drop down between the scapulae, and upon examination a fracture appeared to lie at the margin of the junction of cartilage and bone matrix. This animal was also placed on copper sulfate, at the rate of 3 g. of copper sulfate at 10-day intervals as a drench in water, and the response was extremely rapid.

Both of the calves recovered, and as long as they were kept on copper sulfate they continued to grow. The calf with the broken scapulae eventually reached a condition in which the vertebral column was again in a normal position relative to the shoulder blades, but the animal always had wide withers, indicative of the previous injury. As long as copper was kept in the diet of these animals, they continued to grow with a normal haircoat, normal development of bone and a satisfactory gain in weight that averaged at times better than two pounds per day on the St. Augustine grass.

When copper was removed from the diet of these animals, as it was after six month, the calves started to lose weight, indications of this developing within 30 days. By the end of 90 days, these calves had begun to show a rough haircoat, fading of the haircoat, loss in weight, lowered hemoglobin, and in the first of the two, a broken humerus. Apparently these calves were on a marginal intake as regards their requirement. When copper was again included in the diet, the calves recovered rapidly and went on to reach a weight of about 700 pounds, at which time they were brought to Gainesville from Belle Glade and were placed on radioactive isotope studies (1). It was obvious from the work with these and other calves, repeated over three successive generations (33 animals over 4½ years), that copper had a very beneficial effect on the response of the cattle to the forage. It was intriguing to discover in some of the radioactive work that molybdenum appears to follow the course of phosphorus and was excreted in the milk of the cow, so that suckling calves were probably getting an increased molybdenum intake, compared to the animals with pasture alone as feed.

In older animals, the copper deficiency was somewhat slower in making its appearance, and the usual first indication was a loss in weight, with a beginning of achromotrichia changing the dark red Devons to a light tan, and with lowered hemoglobin values, which often dropped below 50 per cent of the value which we have considered normal (11-12 g./100 ml. blood). These animals were placed on varying levels of copper. Eventually the copper levels ranged from 3 g. of copper sulfate given once in 20 days to a level of 12 g. of copper sulfate given daily. The high level of copper sulfate caused copper toxicity, whereas the low level was barely sufficient to maintain the animals in adequate condition. The interval of 20 days was apparently sufficient for the copper to be dissipated, and recovery was slow. The very low hemoglobin which developed in these animals and their response to added copper appeared to reflect a need for an abnormally high copper intake, inasmuch as the copper values of the forage were marginal. The possible relationship of these nutritional changes to the presence of molybdenum was further complicated by the fact that the molybdenum on these pastures was not extraordinarily high and might be toxic only in regard to its relationship with the copper.

For this reason the phosphorus content was carefully observed, and blood phosphorus and blood phosphatase readings were made on these animals at frequent intervals. Phosphorus in the inorganic blood phosphorus portion of the blood was found to remain at or above normal values and to increase at times when blood phosphatase values increased (2), accompanying lowered copper intake and the development of symptoms of copper deficiency conditions. The seasonal variation which developed in the production of this condition was not marked on our experimental areas, but in some of the commercial herds where similar conditions had been observed, the condition was more likely to develop during the winter.

From the experimental work with cattle on the muck soils, certain things have come to be recognized:

1. On a low copper, comparatively low molybdenum forage, the cattle developed anemia, achromotrichia, anorexia, osteomalacia or rickets, and eventually died.
2. The condition as it developed in cattle was remedied by the administration of copper as a drench or as a fertilizer, although the second method was not always as effective as the first, or by placing copper in mineral mixture.
3. The development of the condition was not correlated directly with the *level* of molybdenum in the forage. There seemed to be a tendency for it to develop in young grass high in molybdenum, as compared to older grass on the same areas.
4. There did seem to be some slight relationship between the season and the development of the condition in cattle.
5. The bone changes were not associated with changes in blood calcium or phosphorus, although in extreme conditions, there seemed to be an increased blood inorganic phosphorus and a possible increase in alkaline blood phosphatase.
6. If the condition was allowed to progress without therapeutic treatment with copper, mortality was high.
7. If the animals were given access to copper at a minimum level, maintained for a short period of time until a rapid improvement in their condition was apparent, and then removed from copper therapy and kept on pasture which was comparatively low in phosphorus, the older animals occasionally developed " falling disease " within a period of 3 to 6 months.

The work with cattle in the Everglades peat or muck areas showed that copper is required in amounts that are considerably above the usual nutritional requirement, and that the increase is probably due to an interrelationship of copper, molybdenum, and perhaps phosphorus metabolism. It was with a view to determining the effect of molybdenum and phosphorus upon copper utilization or vice versa that we have begun and have continued experiments with rats, cattle, and swine, in an effort to delineate the function of copper in the nutrition of these species, and if possible the effects of added molybdenum and of phosphorus on the nutrition of these animals when given various levels of copper. Some of this work has been reported, particularly the use of radioactive isotopes in tracing the elements copper, molybdenum, and phosphorus as they were affected by each other in the nutrition of rats and cattle (1, 4, 5, 6). Continued work with cattle, but on rations that were quite different from those of the St. Augustine grass in the Everglades area, has demonstrated that cattle can withstand rather large intakes of molybdenum without demonstrating any gross change in their body metabolism, but there are definite effects of the molybdenum and phosphorus on copper and vice versa, particularly as regards the rate and route of excretion of phosphorus in these animals.

Where copper is included in the ration at levels which are toxic or slightly below toxic levels, the addition of molybdenum has not resulted in lowered values of liver and blood copper that were significant. Cattle have not had low values of liver copper when high levels of copper were administered with additional molybdenum. When the copper intake was adequate, but not in excess, the presence of additional molybdenum caused a lowered content of copper in the liver, but here again the lowering of the liver copper was not excessive, with levels up to 300 parts per million of Mo in the ration. Where molybdenum has been given in rations that were low in copper, there has been a definite lowering of the liver copper, shown in Table 1 (3, 4). It should be emphasized that the addition of molybdenum to rations that are low or marginal in copper intake does result in lowered copper in the liver and eventually in lowered copper in the blood. In animals that are receiving an excess copper intake, the addition of molybdenum has not served to reduce the accumulation of copper in the liver, and does not appear to be a therapeutic measure for excess copper intake. On the other hand, it should be pointed out that additional molybdenum to a ration with excess copper present results in some increase in the liver molybdenum, and it is not reduced or kept at a minimum level by the extra copper which is present. Furthermore, the presence of molybdenum in a ration that is low in copper or just adequate results in an increased molybdenum content in the liver that may be considered an accumulation.

TABLE 1

Copper Levels in Liver and Blood of Steers with Added Molybdenum and Copper Given by Drench

Group	1	2	3	4
	Liver (biopsy samples at 2 week intervals)			
	p. p. m.	p. p. m.	p. p. m.	p. p. m.
Initial	384.4	350.6	486.3	218.1
Maximum	1468.8	1417.8	486.3	219.9
Minimum	389.4	350.6	107.8	111.9
Max. wk.	8	6	0	10
Min. wk.	0	0	12	6
	Blood			
	γ/100 ml.	γ/100 ml.	γ/100 ml.	γ/100 ml.
Initial	157.5	124.5	106.5	124.5
Maximum	235.0	360.3	234.0	272.0
Minimum	125.4	98.1	106.5	80.4
Max. wk.	15	5	7	3
Min. wk.	8	12	0	8

Daily Treatment

Group 1: $CuSO_4$ daily 5 g.
Na_2MoO_4 daily 1.52 g. 1–9 wks; 3.04 g. 10–18 wks.
Group 2: $CuSO_4$ daily 5 g.
Group 3: Na_2MoO_4 daily 1.52 g. 1–9 wks.; 3.04 g. 10–18 wks.
Group 4: Control.

Max. wk.—Week in which maximum value was obtained.
Min. wk.—Week in which minimum value was obtained.

Thus, we have a condition in which with extra molybdenum and just adequate or slightly above adequate intake of copper, the molybdenum serves to cause a reduction in copper values, but the copper apparently does not interfere with the accumulation of molybdenum in the liver. It has been possible to follow in the whole animal, through liver biopsies, the accumulation and loss of copper and molybdenum in the liver samples, and these values indicate that copper does build up to a maximum plateau, whereas molybdenum accumulates at a much slower rate. Molybdenum does not seem to be accumulative to the same extent as copper.

In these animals, which have been given high molybdenum and copper intakes over seven months, radioactive phosphorus was administered at the time of sacrifice, and an effort was made to study the influence of molybdenum and copper upon the P^{32} excretion of these animals. While the excretion was not abnormal in any case, the pathway of excretion was decidedly changed by the presence of molybdenum in the ration, as may be seen in Table 2. All of these cattle were receiving adequate phosphorus in their ration and had access to steamed bonemeal during the period of these experiments. Similar studies have been carried on with rats, and intensive studies were made on the influence of the elements phosphorus and molybdenum and copper upon each other in regard to the excretion of phosphorus by the animal into the alimentary tract. Some of the results are demonstrated in Tables 3, 4, 5, and 6. It will be seen that the presence of molybdenum and copper in these diets apparently did not change the route of excretion, but markedly affected the rate of elimination. The explanation for the lowered excretion is not certain, but probably is associated with a slight negative balance

TABLE 2

Excretion of Phosphorus in Cattle

Steer No.	Feces			Urine		
	Per cent of Dose	Per cent of Dose per 100 mg.	Total P mg.	Per cent of Dose	Per cent of Dose per 100 mg.	Total P mg.
1	2.32	0.05	4680.0	0.14	0.02	542.0
2	1.97	0.04	4700.0	0.07	0.18	36.0
3	9.44	0.06	15,600.0	0.04	0.14	29.0
4	0.57	0.01	5160.0	2.48	0.11	2300.0

Steers 1, 2, 3 and 4 are from Groups 1, 2, 3, and 4 of Table 1.

TABLE 3

Distribution of Phosphorus-32 in the Feces and Urine of Rats Receiving Added Copper and Molybdenum

(168 hours after administration)

Excrement	Group 1	Group 2	Group 3	Group 4
	35 p.p.m. Cu 80 p.p.m. Mo	35 p.p.m. Cu 0 p.p.m. Mo	0 p.p.m. Cu 80 p.p.m. Mo	0 p.p.m. Cu 0 p.p.m. Mo
	*	*	*	*
Feces	3.05	4.54	5.66	7.74
Urine	12.00	16.10	19.30	26.00

* Per cent of dose.

TABLE 4

Excretion Studies of Labeled Molybdenum Administered to Cattle

(Expressed as per cent of dose per day)

Number of Animals	1		2**	
Age and Weight, in pounds	14 months; 380		9 months; 175	
Mode of Administration	Oral		Oral	
Actual Dosage, g. of MoO_3	7.0		4.6	
Time (days)	Feces	Urine	Feces	Urine
1	1.26	4.83	3.21	0.46
2	9.87	12.65	13.08	2.34
3	9.13	10.92	19.88	7.76
4	6.10	7.50	11.84	4.67
5	5.14	5.26	4.01	2.75
6	1.06	1.30	0.81	1.84
7	0.68	1.20	1.42	0.75
8	0.33	0.52	*	*
9	0.20	0.40	*	*
10	0.14	0.44	*	*
11	0.16	0.16	0.49	0.14
12	0.10	0.03	0.18	0.17
13	0.06	0.01	0.68	0.67
Total for first 5 days	31.50	41.16	52.02	17.98
Total for 13 days	34.23	45.22	55.59	21.55

* Insufficient sample.

** Given drench of 4 g. copper sulfate 45 min. prior to dose.

in the first three groups prior to the P^{32} injection. Preliminary results with swine indicate that molybdenum has a much less marked effect upon the copper nutrition of swine than it does upon herbivorous animals. Swine have been able to tolerate levels of molybdenum up to 1000 parts per million without apparent ill effects for at least 90 days. Still a fourth experiment has been begun in which varying levels of molybdenum are being given with constant but

TABLE 5

MINERAL ACCUMULATION IN LIVER AND BONE OF RATS MAINTAINED ON VARIOUS LEVELS OF COPPER AND MOLYBDENUM IN THE SIMPLIFIED RATION

Group	Animal No.	Copper in the Liver	Molybdenum in the Liver	Molybdenum in the Bone
		p.p.m.	p.p.m.	p.p.m.
1—High Cu (35 p.p.m.) High Mo (80 p.p.m.)	1	33.9	5.3	13.9
	2	47.3	12.3	9.1
	3	28.4	10.9	....
	4	76.3	14.2	....
	5	33.9	12.9	13.8
	6	25.3	....	....
2—Normal Cu (8 p.p.m.) Low Mo (<1 p.p.m.)	1	43.9	1.0	0.1
	2	51.7	1.5	0.1
	3	57.8	1.0	0.2
	4	73.5	2.0	0.2
	5	34.2	1.0	0.3
	6	32.3	1.0	0.1
	7	25.2	2.0	0.2
3—Low Cu (<1 p.p.m.) High Mo (80 p.p.m.)	1*	1.0	6.4	....
	2	14.0	4.0	6.6
	3	22.6	13.2	1.3
	4*	1.0	35.7	12.4
	5	4.5	4.4	10.0
	6	10.4	6.3	9.5
	7	6.0	13.6	12.0
4—Low Cu (<1 p.p.m.) Low Mo (<1 p.p.m.)	1	8.5	2.6	0.1
	2	12.5	1.6	....
	3	3.9	1.1	0
	4	11.8	0.9	1.4
	5	7.3	1.5	0
	6	9.6	1.0	0
	7	11.5	2.1	0.2
	8	3.2	4.6	0
	9	2.6	1.2	0.3

* Animals which died on experimental trial.

TABLE 6

Effect of Dietary Molybdenum on the Accumulation of Ingested Phosphorus^{32} in the Tissues of the Rat
(based on 1 microgram of dose per 100 g. of body weight)

Treatment	Animal No.	Weight G.	Blood		Liver		Kidney		Bone
			Micrograms x 10^4 per g. of fresh tissue	Per cent of total dose	Micrograms x 10^4 per g. of fresh tissue	Per cent of total dose	Micrograms x 10^4 per g. of fresh tissue	Per cent of total dose	Micrograms x 10^4 per g. of fresh tissue
Ration 1	1	199	11	0.78	35	1.51	65	0.55	147
Cu 35 p.p.m.	2	186	13	0.91	115	4.35	74	0.60	183
Mo 80 p.p.m.	3	151	8	0.53	114	4.65	53	0.47	195
	4	167	8	0.58	144	4.90	100	0.70	258
	5	167	6	0.40	60	1.90	28	0.23	79
Average	...	174	9 (±2)	0.64 (±0.16)	94 (±37)	3.46 (±1.39)	64 (±17)	0.51 (±0.13)	172 (±47)
Ration 2	6	172	33	2.34	177	6.45	101	0.75	240
Cu 8 p.p.m.	7	147	21	1.49	162	6.95	115	0.80	361
Mo < 1 p.p.m.	8	182	13	0.89	145	5.70	77	0.50	334
	9	199	19	1.32	140	5.35	120	0.80	423
	10	208	21	1.46	161	6.35	123	0.75	255
	11	184	20	1.42	151	6.55	102	0.65	300
Average	...	182	25 (±6	1.49 (±0.34)	156 (±11)	6.23 (±0.47)	106 (±13)	0.71 (±0.10)	319 (±52)

Rations 1 and 2 correspond to Groups 1 and 2 (Table 5).

marginal copper intakes, in order to study the influence of such a combination upon the reproduction of rats. These results have indicated that molybdenum may cause definite stunting of the young. This work is still in progress, and the final results will not be available for some months.

In discussing and summarizing this work, it may be pointed out that in the herbivorous animal the presence of small amounts of molybdenum, ranging from 2 to 25 parts per million in young growing forage, has been associated with an increased requirement for copper and a changed metabolism of bone, apparently associated with the phosphorus metabolism. Increasing the phosphorus level has not corrected the difficulty which has developed, but additional copper has done so. The possibility of a third factor, one other than molybdenum, cannot be ruled out, inasmuch as much of this work has been done on pastures and not under strictly controlled conditions.

It has been difficult to ascertain whether or not the presence of molybdenum is the sole cause of lowered hemoglobin or the sole reason for the increased requirement of copper. The possibility that a third element is involved or that some particular compound of molybdenum is involved cannot be entirely eliminated. Pointing toward this are some of the results in which higher levels of molybdenum have been given as molybdenum salts without causing changes in the blood picture, and in which forage containing the same amount of molybdenum has caused less trouble when the forage was mature. This would seem to indicate that when molybdenum is present in certain forms it may cause trouble, whereas in other forms it does not. It might also mean that there is present in certain pastures a particular compound which serves to tie up the copper, and that with maturity this compound is changed or bound or decomposed so that it no longer causes difficulty. The possibility that an element such as zinc may be present in critical amounts and cause a similar requirement for copper has not been eliminated.

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DISCUSSION

DR. ARNON: Quite apart from molybdenum toxicity and its relation to copper, do you consider molybdenum as an essential element for animals?

DR. DAVIS: As of this date we have not been able to show that molybdenum is essential. However, in some of the rat experiments which we have under way at the present time it would appear that molybdenum has a very definite influence on the hemoglobin picture. Whether this is due to an increased rate of absorption of iron or copper I am not prepared to say. However, when the molybdenum in the diet is increased to 20 parts per million the reverse seems to be true. How this fits in with Dr. Marston's story I am not sure. Dr. Marston, would you care to comment on the essentiality of molybdenum for animals?

DR. MARSTON: I am not aware of any evidence at the present time which suggests that molybdenum is essential for animals. I think, however, within a few years we may have an answer to this problem. There are hypotheses current which suggest a mechanism by which molybdenum might regulate copper metabolism in the animal, but so far the evidence in support of these is not convincing.

DR. STEINBERG: I believe the Wisconsin group attempted to determine whether molybdenum was essential for animals but was unable to do so.

DR. DAVIS: Yes, that is true. In fact, the Johns Hopkins group has also attempted such studies, but it may be that the molybdenum concentration due to contamination of the diet was sufficient to maintain a normal healthy animal.

Dr. Talbot: Can you describe the situation which applied when " falling disease " became prevalent?

Dr. Davis: We have usually gotten to the ranches after the animals had fallen, and it has been difficult to determine the exact conditions. Our first lead came on the Pierce ranch, where animals had been brought in from another pasture and were placed on this pasture which was apparently lush so far as feed was concerned. The animals came along with a normal growth rate and then developed " falling disease." We took some of our normal mature animals and placed them on a forage low in copper, between two and four parts per million. They were carried on this diet until they were almost ready to die. Then we gave them copper at levels ranging between 0.3 and 0.8 g. of copper sulfate per day at 10 day intervals. They were kept on that for 60 days, and then we stopped the supplement and started them down again; and it was within three to six months after they were put back on the low copper pasture that we observed the following: while a group of the cattle were being driven to the barn one rushed to get away from another animal and fell immediately in its tracks and was dead by the time we got to the animal. Apparently the heart just stopped.

PROBLEMS ASSOCIATED WITH COPPER-DEFICIENCY IN RUMINANTS

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Copper was proven to be an indispensable element for nutrition about 25 years ago, when an investigation of the anemia suffered by animals confined to a diet comprised solely of milk revealed the important role that copper plays in the metabolism of iron. Although the process is well defined, the mechanism which underlies it still remains a mystery to challenge the ingenuity of biochemists. It is, however, but one of a number of metabolic processes in which copper-containing catalysts are involved.

My intention here is not to review the literature of copper metabolism, which is largely devoted to the influence of copper on the utilization of iron, as I shall deal with that task elsewhere. It is my desire, rather, to reveal a few of a large number of fascinating problems that still await solution, and to discuss with you some of what appear to me to be the more cogent findings that have resulted from our studies of the metabolic disturbances which supervene when the quantity of copper available to the animal organism is reduced below that necessary to provide for maximum efficiency of all of its physiological functions.

Our attention became focused on copper metabolism about fifteen years ago, when we realized that the nutritional well-being of sheep is seriously impaired by the inadequate quantity of essential nutrient elements in the pastures which grow over a large proportion of the terrain adjacent to the southern Australian seaboard. These deficient tracts seemingly originated during a period of extreme aridity in post-Pliocene times, when the comminuted marine detritus from an exposed portion of the continental shelf was carried by prevailing winds far inland—in places 100 miles or more—to cover unconformably and at considerable depth the older strata. Subsequent weathering has converted this cover of aeolean sands into a number of soil types, all of the same genesis, and all more or less limited by the same deficiencies. The coastal fringes are composed essentially of shell fragments, windborne into gently rolling dunes, that merge through consolidated limestone ridges—intersected by brown soils derived by leaching from the older dunes—into a hinterland where light siliceous sands imposed upon solonized subsoils prevail over relatively huge areas. All are copper deficient to a more or less degree.

Most of the experiments to which I shall refer here were conducted with sheep on the calcareous littoral tracts where the pastures comprise a very simple plant association, dominated overwhelmingly by the two calciphile grasses *Bromus madritensis* and *Lagurus ovatus*. Our experimental studies established unequivocally that the low cobalt and copper content of these pastures limits seriously their nutritional quality for ruminants. Sheep grazed continuously on this terrain become anemic, and develop a wasting malady from which they succumb, usually within a year; but they will thrive on this deficient fodder if they are provided with an adequate supplement of these metals. Here, then, where the dual deficiency prevails, appropriate treatment of the grazing ruminant will reveal the uncomplicated syndrome of either deficiency.

When a sheep that has been bred on sound country is transferred to these deficient pastures and provided with ample cobalt, it develops the syndrome of copper deficiency, which otherwise would be masked by the more rapidly fatal effects of cobalt deficiency. The first untoward effects appear in the sheep after they have grazed on this deficient terrain for several months, the rate of the onset of the symptoms being determined by the initial copper status of the animals, for depletion of the copper reserves is essentially a slow process.

These reserves, which are mainly concentrated in the liver, are at any time an integrated expression of the animal's nutritional history since its early development, for both the copper in the fetal liver, and the copper in the milk, which provides the early nourishment of the new-born lamb, reflect the copper status of the ewe. In this respect the behavior of copper is distinct from that of iron, for this element becomes concentrated in the fetal liver irrespective of the iron-status of the ewe, and it is almost completely absent from the milk.

In " normal " * circumstances, the concentration of copper in the liver is thus a useful measure of the animal's copper status. Little is known, however, of the physiological mechanisms which determine the absorption and retention of copper. Direct study of the copper balance is difficult, and the findings are uncertain because the quantity of copper that is retained by the animal is such a very small proportion of that ingested in the fodder as to be within the experimental error of the chemical estimations entailed. Segregation of the two components—absorption and excretion—which determine the balance also presents difficulties because copper is secreted into the intestine, presumably via the bile, and only a minute amount—usually less than a tenth of one per cent of that ingested—finds its way into the urine. For this reason it is difficult to assess, by direct methods, the factors which complicate the absorption of copper from the alimentary canal. The fate of copper administered parenterally in moderate amounts suggests, however, that only a small proportion of the copper in the fodder is absorbed, for if copper is injected intravenously almost all of it becomes concentrated in the liver, and, according to the state of the copper-balance, is either retained there or is subsequently excreted slowly into the intestine.

Unlike iron, which is absorbed or rejected by the intestinal mucosa according to the animal's need and which, once absorbed, is lost only in small amounts except by hemorrhage, copper is excreted in a form which is not reabsorbed, and this loss must be restored

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* I have stressed the word " normal," as I shall have occasion later to mention the conditions imposed by the ingestion of other elements which complicate the metabolism of copper. In the deficiency states which then result, neither the concentration of copper in the liver nor in the blood is a reliable guide to the physiological copper status.

from the fodder if the animal is to remain in a normal physiological state for any considerable period. The relative rates of absorption and of excretion at any fixed level of intake depend on the nutritional status of the animal.

When a sheep is confined to copper-deficient fodder, the rate at which it loses copper is influenced by the concentration of copper in its tissues, and by the effective copper content of its fodder. In these circumstances the negative resultant of the copper-balance depends primarily on the concentration of copper in the animal's liver, and so, as this decreases, the rate of depletion falls off exponentially. The converse is also true: when the fodder contains sufficient copper to institute a positive balance, the rate at which copper is retained decreases exponentially as the copper status of the animal improves. Equilibrium is attained by sheep on normal pastures when the concentration of copper in their livers is within a rather wide range, between the approximate limits of 50-300 μg. Cu/g. D.W., for it varies in reflexion of the seasonal flux and wane of the copper content of the available fodder.

The rates of loss and gain of copper by the animal are slow, and in consequence, when the conditions are such as to decrease the normal copper reserves, the onset of the deficiency is gradual. In the course of the relatively slow, progressive depletion of the copper reserves which occurs in mature animals when they are confined to copper-deficient rations, an ordered sequence of distinct symptoms appears, as if, one by one, different metabolic processes become adversely affected, each in its turn, through partial impairment of the particular enzyme system which governs it. No doubt it would be fruitful to study the relative extent to which these enzyme systems fail when the supply of copper available to the organism is no longer sufficient for all of them—conceivably some would suffer more than others in the competition for the inadequate supply. This problem seems to lie at the base of a complete understanding of copper metabolism and of the maladies that are associated with copper deficiency.

It is already amply clear that copper plays an essential part in a diversity of metabolic events. Although a number of enzymes are known to contain copper, and more are suspected, the functions they perform in normal metabolism have not yet been defined.

Our interest in these processes has been devoted more particularly to the part played by copper in the process of keratinization, for a lesion in the wool is the first sign of impaired function that appears during the progressive depletion of the sheep's copper status. In the syndrome of uncomplicated copper deficiency, this lesion reflects the considerable fall in the copper concentration of the blood, a fall which ensues, in the adult animal, long before any other symptom of metabolic disorder becomes manifest. A sheep on the deficient pastures described above, for example, will display in its wool the flagrant consequences of copper deficiency while otherwise apparently in a normal physiological state. These morphological changes in the fleece express in the staple the progressively decreasing efficiency of the chemical mechanisms within the follicle which require copper if they are to function normally. As the animal's reserves wane, the crimp, which is a striking feature of merino wool, becomes progressively less distinct until the fibres emerge entirely devoid of character. The capacity of the follicle to impart crimp returns immediately if the copper concentration of the blood is increased, or if copper is applied directly to the skin.

There is little doubt that the changes in the structure of the fibre, which this lesion expresses, result from a breakdown of catalytic processes in which copper is primarily involved. The follicles of a black sheep depleted of copper lose their ability to impart crimp and their capacity to produce pigment. The role assumed by copper in affecting the transfer of oxygen in the follicle is beautifully illustrated by the reappearance of both crimp and melanin when the normal copper concentration in the follicles is reinstated.

Consideration of the cytology of the follicle leads to the site of this failure. As the proliferating cells at the base of the follicle mature, their expanding protoplasm impels them to present a minimum surface and to fill without interstices the restricted space they occupy, so that each cell becomes faceted and approximates to the shape of Kelvin's minimum tetrakaidecahedron. In this state they are forced towards the conical constriction where they are gradually compressed to about one-eighth of their original cross-section and so become greatly elongated into spindle-shaped cortical cells which, when keratinized, comprise the wool fibre. During extrusion, the globular protein of these cells granulates and proceeds through microfibrillae of fibrous protein (Vörner's trichohyalin of classical histology) to hard keratin.

A specific histochemical reaction with alkaline nitroprusside which forms a deep purple complex with thiol (—SH) groups reveals the process which is impaired by copper deficiency. When this reagent is applied to a normal fibre freshly plucked so as to retain its root, the basal cells are stained very lightly if at all. At the level where the intracellular structures begin to granulate and form into microfibrillae, the staining is intense, and from there, in a normal fibre, it extends for approximately 100μ, to cease quite abruptly in the fully keratinized portion. When applied similarly to a wool fibre from a sheep with a low copper status the intense reaction begins at the same level, but it extends for 1000μ or more—practically the whole of the fibre embedded in the skin reacts strongly, the staining of the upper portion becoming progressively less intense as it approaches the skin surface. On dosing the deficient animal with copper, this greatly increased zone of free thiol groups retracts to its normal position within a few hours.

This evidence leaves little doubt that copper is primarily responsible for catalyzing the oxidative closure of the thiol residues of the prekeratin fibrous protein to the disulphide linkages of keratin. The rate at which this reaction proceeds is a measure of the copper status of the sheep. In the normal integument the oxidation of the reduced fibrous protein is complete in 8 to 12 hrs.—the fibre being set, deep down in the follicle; in the integument of a seriously copper-deficient animal this oxidative change takes 3 days or more to proceed to completion, and so the fibre retains its plastic condition until it emerges at the skin surface.

The preferred molecular orientation in the wool fibre originates during the passage of the cells through the constriction at the base of the follicle. The normal fibre becomes anisotropic during its extrusion through this constriction, and the molecular asymmetry remains. The long-chain fibrous protein molecules apparently are aligned while still in a reduced state and are fixed in this position when, by copper-catalyzed oxidation, the thiol groups of the cysteine residues of adjacent molecules are closed to the disulphide bonds. In the copper-deficient sheep the comparatively slow oxidation of the fibre provides ample opportunity for subsequent disorientation before internal bonding is completed.

The aberrant physical properties of copper-deficient wool—its reduced tensile strength, its lack of elasticity and abnormal stress-strain cycle, its tendency to become permanently set, its reduced affinity for dyes, etc.—with little doubt arise from the abnormal internal bonding and less orderly structure within the fibres. There is no gross change in its amino-acid constitution.

The symptoms which appear after further depletion of the copper status are not so precisely defined as the one that results from a breakdown of oxygen transfer within the follicles, and they may, at least in part, be secondary to the disordered iron metabolism, which becomes manifest as a hypochromic anemia accompanied by the deposition of hemosiderin in the tissues. The severity of the anemia increases until the animal succumbs; but even when the oxygen-carrying capacity of the blood is very much reduced, the anemia itself cannot be indicted for the fatal consequences of the deficiency state, as the dramatic improvement in the general health of the seriously deficient animal which follows immeditely upon treatment with copper precedes any change in the lowered oxygen-carrying capacity of its blood. There is little doubt that metabolic processes other than those responsible for hemoglobin synthesis become impaired in the terminal stages of the syndrome; the cachexia which the animal suffers is due to causes other than a decreased efficiency in the passive transport of oxygen. One is led to suspect a partial failure of the main oxygen transfer system, but even when the syndrome is advanced, the concentration in the tissues of cytochrome C and of its oxidase, although low, is within the normal range found in those of healthy control animals. This system is apparently one of the last to become affected, and probably then its failure is due to the disordered iron metabolism.

The deteriorating copper status which induces the uncomplicated syndrome exhibited by an animal under these conditions, leads to a fall in the copper content of its blood. A sheep transferred from " normal " to " deficient " pastures, where it ingests between 2 and 4 mg. Cu per day, for example, becomes depleted in the course of 6 to 8 months to a stage at which the first symptoms—those of defective keratinization—appear. These become evident after the concentration of copper in the blood has been reduced to less than one third of the normal level of approximately 1.0 μg. Cu/ml. As depletion proceeds, this level falls progressively to < 0.1 μg./ml., and in the interim hypochromic anemia supervenes as a consequence of disordered iron metabolism. This condition progresses slowly. hemosiderin arising from spent erythrocytes is deposited in the tissues, and the concentration of iron in the liver rises steadily from the normal range of 200-400 μg. Fe/g. D.W. to very high levels, which may exceed 35,000 μg. Fe/g. D.W. in the livers of animals that have been confined to a copper-deficient fodder for two years or more. If during gestation the concentration of copper in the liver of a ewe remains below 20 μg. Cu/g. D.W., nervous sequelae are likely to develop in the lamb. These invariably result when the concentration of copper in the ewe's liver remains below 10 μg. Cu/g. D.W.

I shall dwell on these nervous manifestations of extreme copper deficiency because a spastic diplegia in lambs, identical with the demyelinating malady we observe in southern Australia, is enzootic over widely dispersed areas in many parts of the world, and has been described to occur on terrain where the copper content of the pastures—if judged from the relatively few analyses in the literature—is much higher than might be expected to lead to the serious depletion of ewes grazed on them continuously.

The severity of the nervous disorder which appears in copper deficient lambs varies over a range that extends from complete paralysis at birth to a bland ataxia which may make its appearance at any age up to six months or more. Those lambs that are completely paralyzed are usually few among the large proportion of the affected progeny. They never rise, and as they are unable to suckle they are abandoned by their dams and so survive only a few hours. Others, somewhat less seriously affected, are ataxic when delivered. These are able to gain their feet after considerable effort, and some suckle successfully. Their condition usually worsens, however, and few survive longer than a day or so. The incidence of this manifestation is more prevalent. Our observations of experimental flocks indicate that the greatest proportion of the lambs which suffer nervous symptoms is made up of those that develop ataxia during the period when they are between three weeks and twelve weeks of age. These cases are apparently normal when born, although many lack the *joie de vivre* of healthy lambs. With the onset of the symptoms, their gait becomes stiff and stilted, and as the malady develops the movements of the hind limbs become uncoordinated, and the lambs then stagger with an exaggerated swaying of their hindquarters. The severity of these symptoms may progress to a stage of complete paralysis of the hind limbs or may halt at any degree of intensity, according to the amount of copper which becomes available to the lambs. In advanced cases, locomotion for more than few steps is impossible. Even at this stage the reflexes which control the movements of the head and neck are not noticeably affected. The lambs eat with avidity, and when they have consumed the pasture within reach, propel themselves with an uncoordinated crawling motion to another place. Such cases usually succumb to pneumonic infections. A few of the less seriously affected individuals apparently recover if their copper status improves. The obvious ataxia subsides, but its sudden reappearance when the animals are startled proves that the lesions still exist in the nervous system. The behavior of individuals in our experimental flocks has proven that the typical diplegia may develop in aged ewes, which have been depastured for some years on terrain very seriously copper deficient. The manifestation of nervous symptoms arising from copper deficiency in adult sheep, however, is very rare.

Examination of the most prevalent cases—those with uncoordinated gait—reveals a spastic paralysis of the hind limbs. Muscular tone is normal and at times exaggerated. The efferent and final common paths of both the flexion and the extension reflexes are apparently intact; when elicited by the usual stimuli the reflexes proceed with vigor and without confusion. The clinical findings are those which might be expected to supervene on a defect of the upper motor neurones; and the histopathological lesions revealed on examination of the central nervous system confirm this—for the distribution of the characteristic plaques of demyelination in the ventro-lateral columns of the spinal cord that are invariably exhibited is that of the crossed and direct pyramidal paths. The nature of these lesions, and their restriction to the motor paths which serve the hind limbs present intriguing questions to which, at present, there are no satisfactory answers.

Under the grazing conditions we have had occasion to study intensively, ataxia in the lambs invariably and inevitably results when the breeding ewe is very seriously depleted of copper and manifests during the term of her pregnancy all of the other symptoms of the deficiency syndrome. Elsewhere—in Derbyshire for example—ataxia with an identical pathology occurs in lambs from ewes that have been reported to exhibit no particular evident signs of copper deficiency. However, under both conditions of grazing the ataxia may be prevented with certainty by administering copper to the gestating ewe. In the latter circumstances we seem to be confronted with conditions which render the copper in the animal unavailable to the metabolic processes which maintain myelin in a healthy state. It becomes desirable, then, to explore further the factor that complicate the ability of different tissues to utilize copper.

The Influence of Molybdenum on the Metabolism of Copper

The spectacular effect of copper therapy on the intestinal disturbance which supervenes when cattle ingest relatively massive amounts of molybdenum, either as a constituent of " teart " pastures which grow on soils derived from the lower Lias in Somersetshire, or as molybdate added to their fodder, led to a suggestion that the copper metabolism of the grazing animals may be influenced by the molybdenum content of the pasture. While this speculative suggestion should have remained in the realm of ideas, it rapidly hardened into a tacit assumption that the molybdenum content of the fodder is the prime factor that determines the copper status of the grazing animal. It has been assumed that a high concentration of molybdenum in relation to the copper content of the fodder is capable of precipitating the copper-deficiency syndrome in sheep, and conversely that a low concentration of molybdenum in the fodder is the prime cause of enzootic toxemic jaundice, the climax of which is preceded by an abnormal accumulation of copper in the livers of the affected sheep.

The fact that the molybdenum content of pastures is sometimes correlated with the tendency of a sheep grazing on them to accumulate copper in its liver is, in itself, no proof that molybdenum enters the physiological mechanisms involved either in the assimilation or in the excretion of copper by the animal. Soil and climatic factors that favor the concentration of molybdenum within the tissues of certain plants influence greatly the concentration of other elements, and so the molybdenum content of a pasture plant may, by reflecting the conditions in which it developed, indicate an increase or a decrease of the concentration of other elements that may complicate the metabolism of the grazing animal. Circumstantial evidence of this sort is not convincing. There is as yet no experimental proof that additional molybdenum—sufficient to decrease the Cu/Mo ratio of the fodder to that of pastures where ataxia is prevalent—administered to ewes on normal pastures will deplete their copper status to the low level necessary to induce nervous sequelae in their lambs; or, conversely, that additional molybdenum administered to sheep grazed on areas where toxemic jaundice prevails will prevent the accumulation of copper which precedes the hemolytic crises. We have now, however, a considerable body of evidence to prove that the ingestion of molybdate may, in some circumstances, deplete the copper concentration of the tissues, and, in others, may lead to serious interference with the metabolism of copper.

Our current experiments with sheep grazed on terrain where normally they suffer no symptoms of copper deficiency have shown that additional molybdenum will reduce more rapidly the copper concentration of their livers when seasonal conditions are such as to decrease the copper content of the pastures below that necessary to ensure a positive copper balance; and, when pasture conditions improve, will decrease the rate of copper storage materially below that which ensures in the untreated animal. Under these grazing conditions, however, treatment for 18 months with the equivalent of 50 mg. Mo per day has not influenced significantly the concentration of copper in the blood, nor has it reduced the concentration of copper in the liver * of any individual in the experimental flock to a level which is incapable of fulfilling the normal physiological requirements.

These findings are in striking contrast to the results from our experiments with sheep on the calcareous littoral. The evidence from these proves unequivocally that under the nutritional conditions which prevail on these deficient tracts, molybdenum (administered as molybdate equivalent to 50 mg. Mo per day) tends to *decrease* the rate of depletion of the copper from the liver, to *maintain* the concentration of copper in the blood, and *to precipitate sooner, in most flagrant form, the symptoms of copper deficiency.* The later influence of molybdenum may be overcome, for two years at least, by an additional supplement of copper—equivalent to 10 mg. Cu per day—which our previous experiments had shown is necessary to ensure a positive copper balance in sheep on these pastures. The concentration of copper in the blood of the animals treated in this way has remained *very significantly higher* than that of those which have merely been supplemented with copper. However, this quantity of copper superimposed on an intake of molybdenum that has precipitated the symptoms of advanced copper deficiency will lead to spectacular recovery of the animal.

Here is a paradox—the symptoms of the copper-deficiency syndrome, coincident with a concentration of copper in the tissues that in other circumstances would maintain normal function. Clearly, under these conditions, the concentration of copper in the tissues is no longer a measure of the *physiologically effective copper*, for molybdenum apparently tends either to fix copper in a form that is not utilizable by the tissues, or to antagonize, by hindrance, the copper-containing enzymes.

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* As determined by analysis of samples taken by liver biopsy.

This effect of molybdenum on copper metabolism is not confined to sheep. Moderate amounts of molybdenum (1 mg. Mo per day) administered to rats confined to rations which supply just sufficient copper to provide for maximum growth will increase materially the copper concentration of their tissues and precipitate the symptoms of copper deficiency.

It is clear that a partial failure in the assimilation of copper cannot be wholly responsible for the untoward effects which supervene on the chronic ingestion of molybdenum, although evidence from balance experiments suggests that under some circumstances the ingestion of molybdenum favors the retention of copper in the alimentary canal, either by complicating its absorption or by enhancing its excretion into the intestine.

Moreover, the Cu/Mo ratio in the fodder seems not to be essentially significant—there is apparently no stoichiometrical relationship between the amounts of copper and molybdenum that are concerned in these physiological interactions, for in some circumstances at least, a relatively small quantity of molybdenum will bring about a near-maximal effect, and in other circumstances a proportionally small quantity of copper will overcome the untoward physiological effects of a relatively massive amount of molybdenum.

In our experience, molybdenum exerts seriously untoward effects on the effective copper status of sheep only when it is superimposed on a fodder that is incapable of fulfilling their copper requirements. A relatively large amount of molybdenum seemingly exerts no very serious influence on sheep when the fodder is capable of maintaining a positive copper balance.

It is still not clear whether the excessive ingestion of molybdenum by the ewe can lead to the production of nervous sequelae in her lamb; but at the time of writing this paper our experimental groups of ewes that have been treated with the equivalent of 100 mg. Mo per day for two years have been mated. If these produce ataxic lambs, then it will appear as if molybdenum can exert a preferential effect on the copper-containing enzymes of the metabolic processes which maintain the myelin of the motor tracts in a healthy state. Pending direct evidence that such experiments may provide, it will remain doubtful whether the ingestion of molybdenum has any etiological significance in the development of the nervous disorder which leads to ataxia in lambs. The factors responsible for outbreaks of enzootic ataxia in flocks confined to areas where, it has been claimed, the copper concentration of the pastures is high, apparently operate more effectively than molybdenum. It is perhaps desirable to extend the search for other factors in these pastures which may influence the copper metabolism of the grazing animals.

DISCUSSION

Dr. GRANICK: Is there a lack of copper in the soil on these sandy regions near the sea? It would seem to me that there should be some copper even in this region.

Dr. MARSTON: These soils are by no means completely devoid of copper. They are relatively short of it, and what is there is apparently not readily assimilable by most plants. Hence the natural vegetation is restricted to a few species capable of completing their life cycles in this difficult environment.

Dr. VALLEE: Is that sandy soil underneath the terrain on which the copper-deficient plants grow?

Dr. MARSTON: The aeolean sands of which I have spoken cover the older strata to considerable depth — from 10 to 100 feet or more.

Dr. COMAR: Does the application of copper to the soil improve vegetation?

Dr. MARSTON: Yes. Application of manurial dressing of copper sulfate elicits remarkable responses. My colleague D. J. Riceman has shown that certain legumes (*Medicago sativa*, for instance) which fail completely on this terrain without additional copper, grow luxuriantly when the land has been dressed previously with the equivalent of 7 lbs. of $CuSO_4 \cdot 5H_2O$/acre. Species, and even strains of the same species, differ remarkably in their ability to gather their requirements of copper from this terrain. On the untreated virgin soils rye-corn does well, whereas the mulga and aegerian varieties of oats fail to head but will produce good crops if the land has been previously dressed with soluble copper salts. The Brandenburger variety of oats, however, does well on the virgin soils and responds very little to additional copper. The grasses *Lagurus ovatus* and *Bromus madritensis*, which overwhelmingly dominate the natural pastures, grow well without additional copper and respond but little, if at all, to copper-containing manurial dressings, although such treatment will enhance materially the copper-concentration in their tissues. Clearly, these phenomena reveal physiological problems that merit attention.

Dr. COMAR: What is the approximate copper content of the plants grown on this soil?

Dr. MARSTON: The fodder plants which develop naturally in this terrain are restricted to two calciphile grasses. The copper content of these varies between 2 μg. and 4 μg. Cu/g. dry weight according to their stage of growth.

Dr. NELSON: What is the pH of the soil?

Dr. MARSTON: The reaction of the soil is quite alkaline — about pH 8.3 in the top 9 inches, for the soils consist essentially of marine shell fragments.

Dr. NELSON: Does this affect availability?

Dr. MARSTON: Perhaps to some plants — *Trifolium subterraneum,* for example, will not develop there and will exhibit deficiency lesions even when provided with heavy dressings of copper salts — but apparently not for all, because the effects of a manurial dressing of copper sulfate on those plants which grow there only after additional copper has been provided persists for several years, the response tending to increase rather than decrease with the lapse of time.

Dr. CUNNINGHAM: We have found no defect in the wool in New Zealand which could be attributed to copper deficiency.

Dr. MARSTON: The lesion in the fleece that so strikingly proclaims a state of copper deficiency in the Merino sheep is a visible expression of a profound change in the structure of the fibres brought about by an impaired rate of conversion of prekeratin to keratin. It thus depends on at least two variables, viz., the degree of copper depletion, and the rate of cell proliferation within the base of the follicles, the latter being determined by the over-all nutritional state of the animal. Alteration of the crimp may not so easily be discerned in the fleeces of sheep which lack the propensity to impress a marked crimp in their wool; but the rapid reversion to the true hereditary type of wool in the fleece grown subsequent to the renewal of the normal copper status in a deficient sheep is an invariable phenomenon. In our experience, the relatively poor crimp in the wool of Border-Leicester sheep will disappear when the animals become copper deficient, and reappears dramatically, like that of the Merino, on renewal of a normal copper status.

Dr. FUHR: Has the wool been examined by x-ray diffraction analysis? If so, what does that show?

Dr. MARSTON: Yes, the wide angle diffraction patterns from the "normal" and copper-deficient ends of fibres taken from sheep that have been treated with copper after being allowed to become deficient are not obviously different — both are identical with Astbury's α-keratin and both indicate a similar degree of asymmetry with a periodicity of 5.1 Å. But one would expect little difference in the shorter spacings — indeed, there is other evidence to show that these are unaffected. The degree of disorientation might be assessed from the low angle scattering, which in the future might be measurable when suitable apparatus is developed. Other physical measurements — the abnormal stress-strain cycles characteristic of the copper-deficient fibres, for example, — strongly indicate a gross disorder of that alignment of the long-chain molecules which is a feature of the normal Merino wool fibre.

Dr. GRANICK: What do you feed the animals in the winter months?

Dr. MARSTON: The sheep are on the pastures continually. They receive no supplementary fodder.

Dr. VALLEE: Has the incidence of neurological disease in humans been studied in these various deficient areas in Australia, New Zealand, and England?

Dr. MARSTON: Not in so far as I am aware. Demyelinating maladies are not particularly prevalent in Australia, and there is no evidence that their incidence is high among humans inhabiting the areas where grazing stock suffer the effects of copper deficiency. The cause of the relatively high incidence of nervous sequelae among the human population of Great Britain is, I believe, being sought by medical authorities there.

COPPER AND MOLYBDENUM IN RELATION TO DISEASES OF CATTLE AND SHEEP IN NEW ZEALAND

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In New Zealand, work on copper deficiency had its inception in the study of problems associated with the farming of land reclaimed from peat swamps. Some 250,000 acres of such land have been reclaimed and, when properly drained and consolidated, have proved to be fertile, and capable of growing good improved strains of pasture. The nutritive quality of the pasture, however, is not equal to its appearance, and the cattle population it supports is unthrifty and poorly producing. Paradoxically enough, each springtime when the pasture is at its best the animals are at their worst, for at that season they become afflicted with an acute, persistent, and debilitating scouring. So characteristic is the scouring and so closely associated with peat areas that " peat scours " has become the popular name for the disease in cattle. Management practices have failed to improve the health of stock; pasture improvement has merely increased the severity of the scouring disease; grading up of stock has aggravated susceptibility. All in all, the farmer on this type of land has been seriously handicapped. His unthrifty stock produced him small returns and his attempts to improve farming methods were frustrated by further deterioration of his cattle. In the past many farmers struggled along at a low economic level, and some were forced to give up their farming venture at considerable financial loss.

The first hint that a copper deficiency might be involved came from the observation that bluestone and nicotine sulphate drenches afforded some temporary benefit to scouring cattle. These drenches had been employed in the belief that the scouring was caused by internal parasites. Enquiry showed, however, that the scouring was in no way associated with parasitic infestation. Apparently the benefit derived by the cattle arose from some material supplied by the drench, and the hypothesis was formed that the beneficial factor was copper. In pursuing this line of enquiry it was found that peat pastures and the animals grazing on them did, in fact, contain less than normal amounts of copper. It was found, further, that regular supplies of copper to animals would actually bring about permanent relief of disease symptoms and an increase in productiveness. These findings provided a *practical* solution to the problem of the peat lands.

But already it was suspected that a simple deficiency of copper in the soil, pastures, and finally in the animals, was not the whole story. On some peat lands severely deficient in copper, cattle did not become affected with peat scours. They were unthrifty, and not normal healthy animals, but they did not have the characteristic seasonal diarrhea of peat scours. It appeared that there must be two types of disease in cattle, both associated with copper deficiency but showing different symptoms. Both could not be due solely to a deficiency of copper. Research showed that uncomplicated copper deficiency did not, in fact, cause peat scours. In this disease another factor was implicated, aggravating the copper deficiency and intensifying the symptoms of disease. This complicating factor, on present evidence, is an excess of molybdenum.

Cattle are not the only animals affected by copper deficiency. Sheep also are susceptible, and the field of investigation has been widened to include a study of the occurrence and control of a disease which affects the young of this species on copper-deficient land.

Again, the peat soils prove to be only one of several soil types in New Zealand which are deficient in copper. A deficiency occurs also in consolidated coastal sands and in other leached sandy soils, in marine silts, and in some river silts, and the total area of these soil types is greater than the area of peat soils.

It is my purpose in this paper to describe the diseases in stock in New Zealand which are associated with a deficiency of copper and with an excess of molybdenum, and to present evidence for this correlation. Evidence and conclusions relating to copper will be more briefly touched upon, since these are of earlier date and much has already been published. For molybdenum more detail is presented, since most of the results in this connection are as yet unpublished.

SYMPTOMS OF DISEASE

The classes of stock kept on the areas with simple copper deficiency are sheep, beef cattle, and occasionally dairy cattle, while on areas with complicated deficiency the stock are almost entirely dairy cattle. Most of the sheep are of the romney breed, the beef cattle usually aberdeen angus or herefords, and the dairy cattle are predominantly jersey. The practice is to keep stock all the year round grazing on permanent pastures of clovers and grasses and to supplement winter grazing of cattle by feeding hay saved from surplus spring growth of pasture.

CATTLE

Simple copper deficiency. Adult cattle are anemic, unthrifty and poorly grown, their reproductive capacity is reduced, and the coat color of some red animals changes to a bleached yellowish red. The growth of calves is slower than normal, their bones fracture easily, and some cases of ataxia have been observed. The ataxia develops after exercise, usually after a run of about 30 to 50 yards, and takes the form of sudden loss of muscular control of one hind limb with the result either that the animal falls on one side with the leg extended or the rump swings round to the ground and leaves the animal in a sitting posture. After a rest, the power of slow locomotion is regained. Fig. 1A shows typical attitudes in an ataxic heifer.

Peat Scours—Complicated copper deficiency. Calves are difficult or impossible to rear because of unthriftiness, debilitating spring

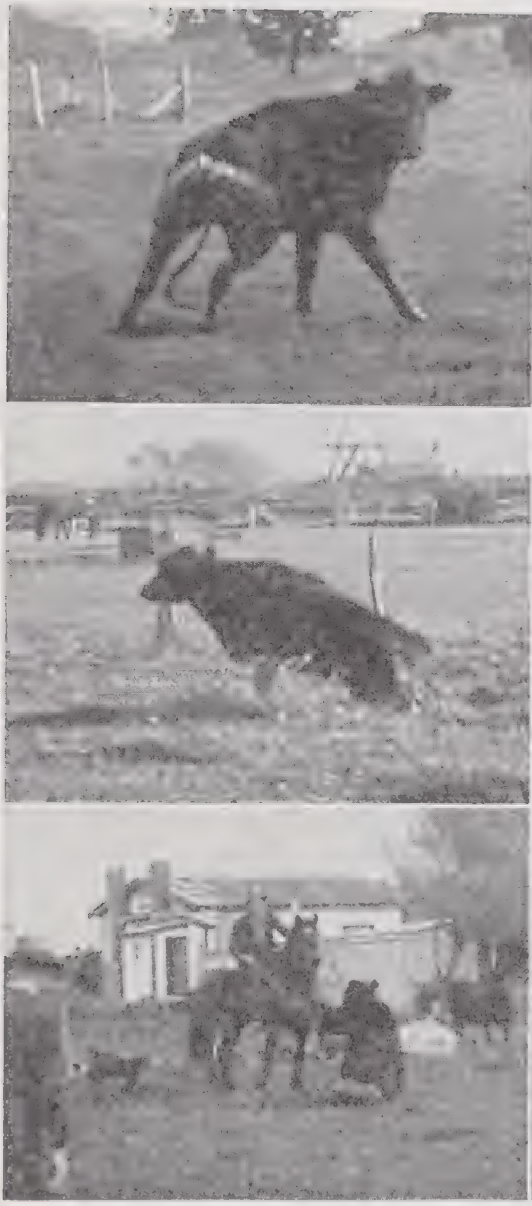


FIG. 1A

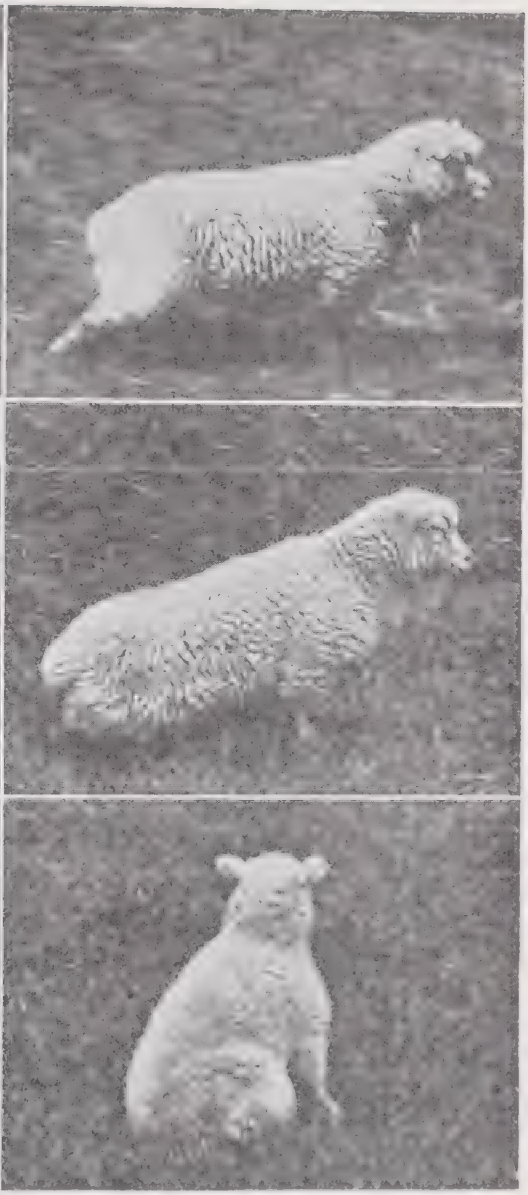


FIG. 1B

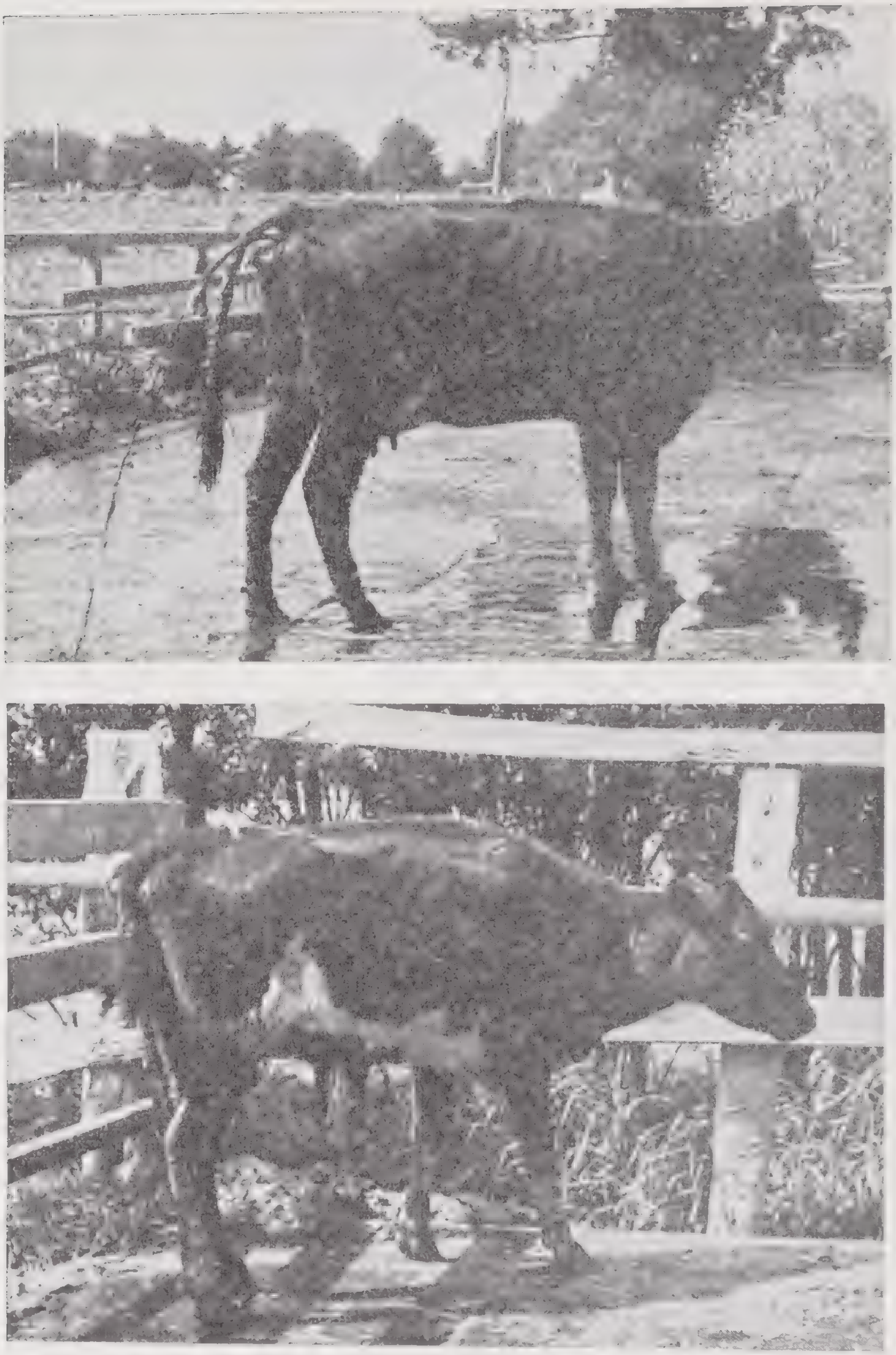


FIG. 2

scouring, increased susceptibility to internal parasites, and proneness to fracture of the bones.

Adult cattle are anemic and debilitated, the coat is rough and staring, and the color of black animals becomes tinged with dull red or is flicked with gray and, especially, becomes gray round the eyes. The animals scour profusely and persistently on flush pastures of spring, and to a less extent of autumn, and at these times become extremely debilitated and produce very much less meat or milk. During the summer months, when scouring ceases, the cattle regain condition to a considerable extent and recover in milk production, but do not reach the yield in meat or milk that could reasonably be expected from the fodder supplies. Some of the more debilitated animals die in the scouring reason, but deaths are not numerous and usually result from some secondary cause.

The only objective sign distinguishing complicated from simple copper deficiency is the acute seasonal scouring and the seasonal recovery in condition. Fig. 2 shows typical cases of peat scours.

SHEEP

Adult sheep of both sexes are healthy in spite of marked depletion of copper, a depletion which results from prolonged subsistence on copper-deficient pastures. The wool of the romney is normal and shows no change comparable with the "stringy" appearance described in Australia (1).

Characteristically, the effects of copper deficiency of the ewe are seen only in the lamb, which may be affected by an acute osteoporosis predisposing to fractures, or by an ataxic condition associated with regional degeneration of the myelin of the nervous system.

The osteoporosis appears to result from a less pronounced depletion of copper than is necessary to cause ataxia, for if a mob of healthy ewes well stocked with copper is put onto deficient pasture, a high incidence of fractures occurs in lambs dropped in the second season, whereas in the third and subsequent seasons most of the lambs affected suffer from ataxia.

Ataxia develops only in lambs between the ages of 3 weeks and

4 months and is then permanent. The symptoms are the same as those described for milder cases of enzootic ataxia in Australia (2) and swayback in Great Britain (8), but acute cases associated with brain cavitation do not occur. Some of the postures in ataxic lambs are depicted in Fig. 1B.

Ataxia in lambs has occurred on areas where complicated copper deficiency occurs in cattle as well as on those where simple copper deficiency occurs.

OTHER ANIMALS

Adult horses thrive on deficient areas where cattle and sheep are affected. This may be due in part to the use, as supplementary feed, of chaff and oats imported from other areas. There have been some reports of ataxia in pigs but opportunity to examine cases has not occurred.

EVIDENCE FOR COPPER DEFICIENCY

This is presented under two headings. The first gives the chemical findings on soils, pastures, and on the tissues of affected animals; the second is a consideration of the effect of increased copper supplies to animals.

SOILS, PASTURES AND ANIMAL TISSUES

A general statement of the copper status on areas of simple and complicated copper deficiency is shown in the following table (Table 1). These data have been considered in more detail in previous publications (4), but the summarized form presented is sufficient to illustrate the main features of the findings.

Total soil copper is by no means a measure of the copper that is available to plants and is, therefore, much less significant from the point of view of animal nutrition than is pasture copper. In consequence, greater emphasis has been placed on pasture than on soil examinations, and the mean values quoted for soil in Table 1 refer to only some 20 samples. The figures indicate, nevertheless, that the total copper in peat soils is much lower than in normal mineral soils. "Available" soil copper, measured by the *Asper-*

gillus niger technique (9), is regarded by some as a suitable method of assessing the copper status of a soil. Wright and Johnston (11) have commenced a study of New Zealand soils by this technique. They have found that results for organic soils are unreliable but those for mineral soils correlate reasonably closely with the copper content of pastures grown on the soil. Deficient sands are found to be very low in available copper and give results of from 0.1 to 1.6 parts per million, whereas normal mineral soils are much higher, and some contain as much as 22 parts per million.

TABLE 1

MEAN VALUES FOR COPPER CONTENTS OF SOILS, PASTURES, AND ANIMAL TISSUES

			Animal tissues					
	Soils	Pastures	Sheep			Cattle		
	Total Cu, p.p.m. in dry matter	p.p.m. in dry grass	Blood mg./100 ml.	Liver		Blood mg./100 ml.	Liver	
				Lamb	Adult		New-born	Adult
				p.p.m. in dry liver			p.p.m. in dry liver	
Normal	18	11	0.09	200	350+	0.09	380	200
Copper-deficient sands	..	3	..	..	..	..	..	10
Peat soils:								
simple copper deficiency	..	3	0.02	8	20	..	..	5
complicated Cu deficiency	5	7	..	..	..	0.05	55	9

The figures for pastures and animal tissues from peat soils represent the results of examination of very large numbers of samples. They can therefore be accepted as giving a true picture of the situation as it occurs in New Zealand. For the copper-deficient sands, on the other hand, the figures are less numerous, and the mean values presented must at present be regarded as tentative.

The copper in pastures grown on deficient sands and on the peat areas with a simple deficiency is unmistakably low when compared with the normal, whereas pastures from areas with " complicated " deficiency show a relatively mild degree of deficiency. Bloods and livers of animals on all types of deficient area show marked deficiency, and what is particularly apparent is the similarity of depletion despite the differences in copper intake. The significance of this will be considered later.

Prevention or Cure by Increasing Copper Intake of Animals

On areas of either simple or complicated deficiency, diseases in animals are effectively controlled by increasing copper intake.

For cattle a treatment that temporarily controls the scouring of peat scours is an oral dose of 3.5 g. of copper sulphate, and if this is repeated one per week control is maintained. Symptoms in cattle are prevented by supplying licks containing 2 per cent copper sulphate, by adding copper sulphate to drinking water, or by spraying copper sulphate onto hay, the object of these methods being to supply each head of cattle with about 3.5 g. of copper sulphate each week throughout the year.

In the case of sheep, New Zealand experience has agreed with that in other countries in showing that ataxia in lambs, once developed, cannot be treated, but that prevention can readily be achieved by supplying copper to the mother before parturition. For example, a weekly dose of 1.5 g. of copper sulphate administered to the ewe throughout the gestation period is effective; so also is the same treatment administered for the last seven weeks before the lamb is born. A more restricted program of three doses of 2.5 g. each given to the ewe at fortnightly intervals about the middle of the gestation period also prevents all symptoms in the lamb, but this method is not suitable for general use, as 2.5 g. of copper sulphate is near a toxic dose for small ewes. It has been found also that ataxia does not develop in susceptible lambs if they are dosed regularly twice each week from birth onwards, with 35 mg. of copper sulphate in each dose.

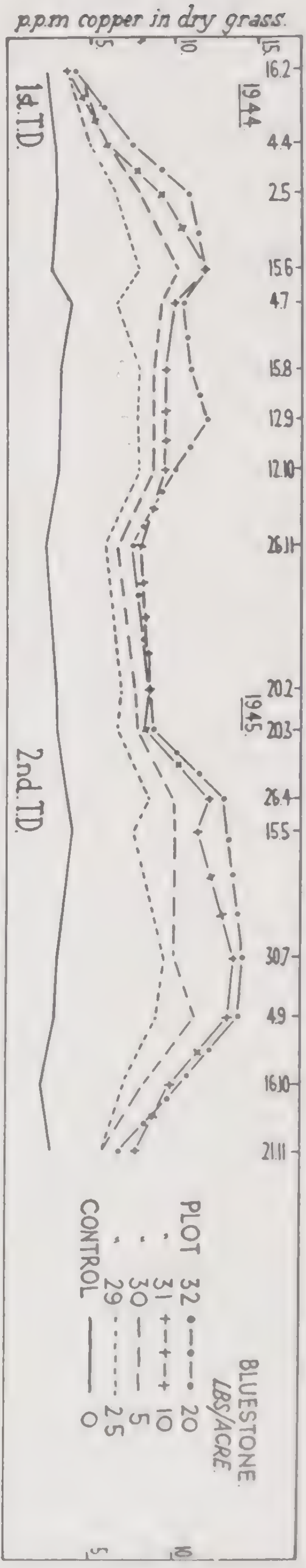
The most effective, simple, and cheap method of controlling copper deficiency diseases in cattle and sheep is to topdress the pastures with copper sulphate, using 5 pounds on each acre each year in the autumn topdressing (5). The copper sulphate can be applied alone or mixed with any of the usual agricultural fertilizers, the most common form being a commercially produced copperized superphosphate containing 56 pounds of bluestone per ton. Some areas of land are too rough for fertilizer-spreading machinery, and for these it has been shown that a distribution of powdered sulphate from aircraft is economic and effective (1).

Supplying copper by topdressing the pastures

Results of some of the experimental work on this aspect are depicted in Figs. 3 and 4, which summarize the response in copper content of pasture to topdressing with commercial bluestone at the rates of 0, 2½, 5, 10, and 20 pounds per acre.

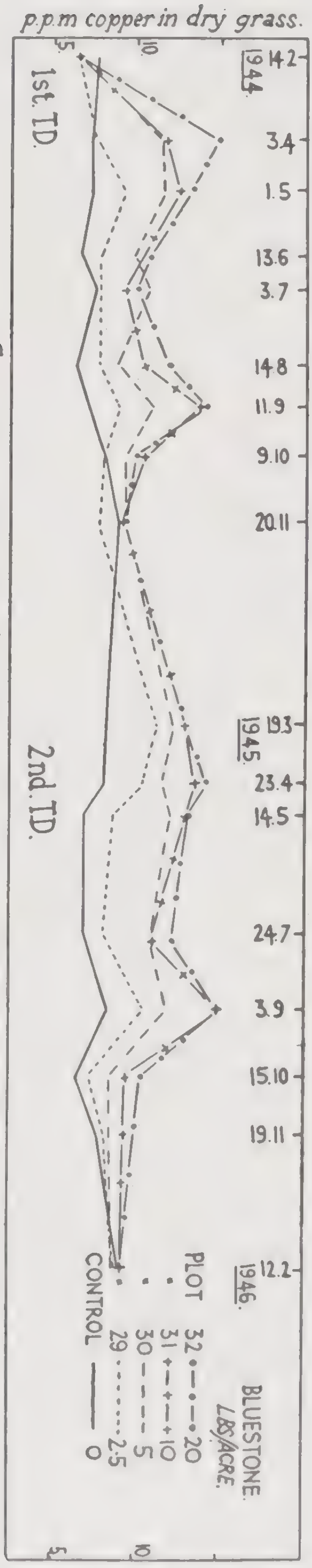
Fig. 3 refers to a series of plots on simple copper-deficient peat land, and Fig. 4 to a similar series on complicated copper-deficient peat areas. It is apparent that in both cases an annual dressing of 5 pounds of copper sulphate per acre brought the copper content of the pasture close to 10 parts per million for most of the year, and that topdressing for two successive years did not result in a dangerously high level of copper in the pasture. Subsequent annual topdressings on these same plots have shown that topdressing for 5 successive years produces a picture almost exactly the same as shown above. In other words, annual topdressing with 5 pounds per acre of copper sulphate is a safe and satisfactory procedure for peat lands.

The work on plots has been amplified by numerous paddock-scale and farm-scale topdressing experiments with normal stock-grazing procedure, and these experiments together with the experience of many farmers over a number of years have confirmed the safety of this rate of topdressing and its efficacy in controlling disease and increasing production in animals on peat land.



Copper in pastures topdressed with bluestone - *Simple deficiency area.*

Fig. 3



Copper in pastures topdressed with bluestone - *Complicated deficiency area.*

Fig. 4

MOLYBDENUM AS A FACTOR IN COMPLICATED COPPER DEFICIENCY

Peat scours is distinguishable from simple copper-deficiency disease by a separate enzootic incidence and by the severe seasonal scouring of affected animals. Clearly some factor additional to copper deficiency is involved in the cause of peat scours. The similarity of the scouring symptom to that in "teart" of Somerset (7), known to be associated with excess molybdenum, suggested that here also molybdenum might be playing some part.

In investigating this possibility it was found that molybdenum does occur in more than a "normal" amount in peat-scours pastures, especially, as Fig. 5 shows, in the season of the year when scouring symptoms in cattle are most marked. The molybdenum level in the pasture is, however, considerably lower than that in teart pastures. It was necessary, therefore, to determine whether the amount of molybdenum present would cause scouring and whether it could in addition be responsible for other characteristics of peat scours, such as reduced gains in weight and susceptibility to bone fractures in young cattle, and depletion of liver stores of copper in old and young.

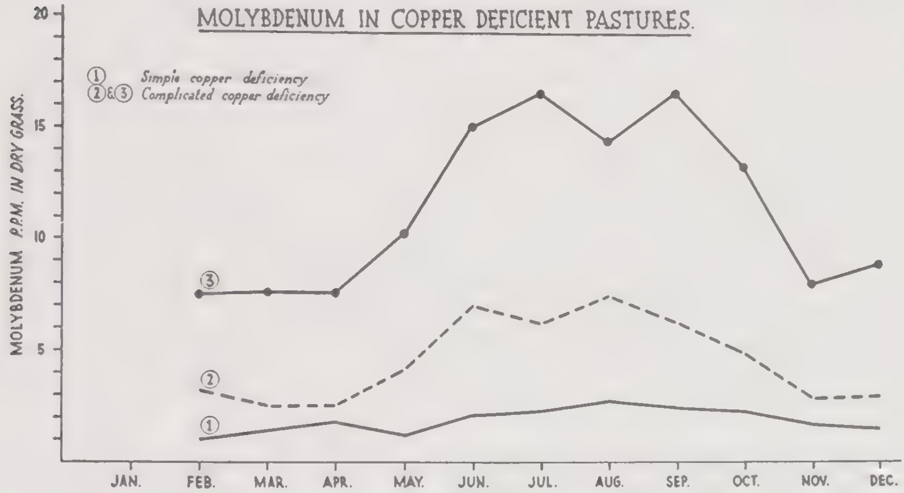


FIG. 5

In respect to depletion of copper in the livers of cattle, reference to Table 1 will show that an unexpectedly low amount of copper in the liver is a characteristic of the complicated deficiency disease. To quote from that table, the mean pasture and liver contents for simple deficiency areas are 3 and 5 parts per million respectively, for complicated deficiency areas 7 and 9 parts per million, and for normal areas 11 and 200 parts per million. An exact parallel between dietary copper and liver copper would not be expected, but a much closer agreement would be anticipated than is shown by the figures quoted. The low value for liver copper in the complicated deficiency suggested that some agent had depressed its assimilation or storage, and experimental work to be quoted below will show that increased dietary molybdenum can exert such an effect on copper metabolism.

Sheep as well as cattle have been studied under conditions of increased molybdenum intake in an investigation of the occurrence of ataxia that occurs in lambs on complicated deficiency areas. On such areas the copper in the fodder would appear to be sufficient to meet the requirements of the sheep, and the occurrence of ataxia can mean only that some factor has interfered with the availability of copper to the ewe or to its lamb. The possible role of molybdenum in this connection has been examined.

Molybdenum Feeding to Cattle

Experiments have been carried out on pastures normal in copper content (i. e., containing about 10 parts per million of copper) and on pastures containing half or less than half this quantity of copper. Molybdenum intake was increased by drenching with solutions of ammonium molybdate three times a week or by topdressing pastures with the same compound. The cattle used were, in the main, yearling heifers at the commencement of an experiment, although in some cases, where very high doses of molybdenum were administered, aged dairy cattle were employed.

An example of the effect of extra molybdenum on a basal diet of pasture which contained 10 parts per million of copper and 1.5 parts per million of molybdenum is an experiment at Wallaceville conducted from Aug. 1, 1945 to Feb. 19, 1947. Two groups of

5 heifers each were depastured together, and molybdenum was administered to one group. The amount was 300 mg. per dose or 900 mg. per week at the start, and as the animals grew and ate more, this was increased to 1800 mg. per week, so that the concentration of molybdenum in the food remained at about 30 p.p.m. At the end, after 81 weeks, the liver and blood composition was as follows:

	Liver		Blood	
	p.p.m. in D.M.		mg./100 ml.	
	Cu	Mo	Cu	Mo
Treated animals	10	5.4	0.04	0.06
Untreated animals	96	3.8	0.09	0.006

Scouring did not occur at this level of molybdenum intake but was marked when the amount employed was doubled.

TABLE 2

EFFECT OF DRENCHING MOLYBDENUM AND COPPER UPON YOUNG CATTLE ON A LOW COPPER DIET

Period, Aug. 1, 1945—Dec. 8, 1945. 17 weeks.

		Drenched 3 times per week	Liver (Dec. 8, 1945)			Blood (Oct. 17, 1945)		
			p.p.m. on dry liver			mg./100 ml.		g. % Hb
			Cu	Mo	Fe	Cu	Mo	
Group	1	nil	5.7	5.3	1940	.015	.013	8.3
(15 ind. per group)	2	Mo 150 mg.	6.5	26.1	2970	.015	.065	7.6
	3	Cu 100 mg.	60.6	3.8	565	.082	.016	11.2
	4	Mo 150 mg. Cu 100 mg.	49.7	7.4	683	.072	.077	11.0

Two experiments on pastures of low copper content will be described. In the first, 4 groups of 15 heifers each run on pasture which contained 3 p.p.m. of copper and 2 p.p.m. of molybdenum were dosed with solutions of molybdenum, copper, or molybdenum and copper in the amount shown in Table 2 for a period of 17 weeks

Analytical determinations made in blood about half way through the experiment and on liver at its end are shown in the table.

No facilities for weighing were available at the time of this experiment, so that body weights cannot be given. As judged by appearance, the heifers in groups 3 and 4 were in very good condition and grew much better than those in groups 1 or 2. All animals in the latter groups were thin, and were rough in coat, and the group 2 animals had persistent diarrhea.

Actual growth rates and data for liver content for a second experiment are summarized in Table 3. This table refers to 12 heifers grazed on an area that had been topdressed with 5 oz. of ammonium molybdate per acre the year before, and the pasture contained 5.5 p.p.m. of copper and 18 p.p.m. of molybdenum. Six of the heifers were dosed three times per week with copper sulphate, or 20 mg. of copper per dose.

TABLE 3

COPPER SUPPLEMENTS TO YOUNG CATTLE ON A LOW COPPER, HIGH MOLYBDENUM PASTURE

Period, April 28, 1948—Jan. 27, 1949. 39 weeks.

	No. of animals	Treatment 3 times per week	Liver Jan. 27, 1949 p.p.m. in dry liver		Body weight	
			Cu	Mo	April 28, 1948 lbs.	Jan. 27, 1949 lbs.
Group 1	6	nil	7.6	7.4	315	318
Group 2	6	20 mg. Cu	9.7	4.1	314	425

In this experiment and in the one summarized in Table 2, an increased molybdenum intake reduced growth rate and induced scouring and unthriftiness similar to that in young cattle on peat scours land. As in natural cases of peat scours, extra dietary copper controlled scouring and improved growth.

Prolonged feeding of molybdenum to cattle on a normal intake of copper depressed the storage of copper in the liver. This effect was less pronounced when the dietary copper was very low, and when the liver copper was already low from this cause, but it has been confirmed repeatedly in cases where the copper in the diet is near the normal level.

It has been found, as illustrated in Tables 2 and 3, that increased molybdenum in the food causes an increased storage of molybdenum in the liver. Feeding extra copper with the same molybdenum intake decreases the amount of molybdenum in the liver. A reciprocal effect therefore exists between molybdenum and copper, and each element has the effect of reducing liver storage of the other. Molybdenum has also been found to accumulate in the kidneys and spleen under conditions of increased molybdenum intake, and this accumulation was not under the same control by copper as liver storage.

From Table 2 it is seen that in copper-deficient heifers the hemoglobin content of the blood was lower than in normals and the iron content of the liver higher. Extra molybdenum further depressed the hemoglobin and increased the liver iron. Added copper reduced the liver iron and increased the hemoglobin.

Brittleness of Bones

Seven of 27 copper-deficient heifers and 16 of 33 copper-deficient heifers which received molybdenum supplements suffered fractures of the long bones, usually of the humerus. Of 52 similar heifers in the same experiments and running on the same paddocks but which received copper, none had fractured bones. The fractured bones showed a very mild degree of osteoporosis but were virtually normal in appearance and shaft thickness and showed no macroscopic defect that would account for their apparent brittleness. Blood calcium and phosphorus were normal. The reason for the high proportion of fractures has yet to be found, but the observations are of interest to the problem of peat scours, since many calves on peat scours land have fractured bones. The high incidence of fractures in copper-deficient lambs is also of interest in this connection. A definite osteoporosis has been observed in such lambs, and the mechanism which gives rise to this also awaits explanation. Neither adult cattle nor adult sheep on copper-deficient pastures show bone weakness, but an acute lameness, unaccompanied by any demonstrable lesions, occurred in cows given very high doses of molybdenum for long periods.

MOLYBDENUM FEEDING TO SHEEP

In an experiment at present in progress at Wallaceville, 12 normal ewes have been drenched with 150 mg. of molybdenum three times per week since Oct. 31, 1945. The pasture copper is 10 p.p.pm. and molybdenum 1.5 p.p.m.: with the added molybdenum the content in the dry matter of the fodder would be approximately 30 p.p.m. All of the ewes survived through three lambing periods, but 4 died in 1948 from accidental causes, so that there were 8 on experiment in 1949, and 8 remain for the forthcoming lambing. A summary of the results so far obtained is shown in Table 4. In Fig. 6 are drawn the mean values for copper on biopsy samples for ewes and individual values for liver copper of new-born lambs. Liver biopsy samples taken at long intervals from the ewes show a steady drop in liver copper. Between July 24, 1946 (after the experiment had been in progress for 9 months) and March 23, 1948, the mean liver copper dropped from 575 p.p.m. to 103 p.p.m. The drop was rapid at the start and has been negligible during the past 15 months, which would suggest that around 100 p.p.m. of copper in the liver is a balance between the avidity of the tissue for copper and the depleting effect of the amount of molybdenum fed.

The treatment of the lambs has been different in the different years. In each year except 1947 some of the lambs were killed at birth for determination of their liver copper and molybdenum. In 1946, the surviving lambs were drenched from 2 weeks of age until weaning at 5 month old with one-fifth the dose of molybdenum given their mothers. At weaning they were slaughtered and their liver copper and molybdenum determined. In 1947 the lambs were left on pasture with no special treatment and were observed for symptoms of ataxia; no liver determinations were made. In 1948 and 1949, the lambs were housed from birth and given access to a diet of hay and grain of low copper content as well as their mother's milk, the mothers being allowed on to pasture for a period in each

TABLE 4

Molybdenum Feeding to Breeding Ewes on Normal Copper Intake (Oct. 31, 1945—Still Proceeding)

Blood	Oct. 31, 1945	Mar. 1, 1946	April 26, 1946	July 24, 1946	Aug. and Sept., 1946	Feb., 1947	Mar. 24, 1947	Aug. and Sept., 1948	Dec., 1948	Nov. 3, 1948	Dec. 7, 1948	Aug. and Sept., 1949	Dec., 1949	Mar. 23, 1950
Hb. g. %	11.1	12.0	11.1	..	..	..	..	..	..	..	..	..	..	10.4
Cu mg./100 cc.	0.07	0.10	0.12	..	..	..	..	..	..	0.08	..	..	..	0.08
Mo mg./100 cc.	0.006	0.34	0.05	..	..	..	..	..	..	..	..	..	..	0.24
Ewes Liver Biopsy														
Cu p.p.m.	..	..	..	575	..	..	301	..	..	..	106	..	..	103
Mo p.p.m.	..	..	..	22	..	..	26	..	..	..	31	..	..	32
Lambs—At birth Liver														
Cu p.p.m.	..	..	..	(9	52 13.3 to 103	..	(10	69 11 to 138	..	..	(6	6.3 5.4 to 7.9	..	..
Mo p.p.m.	..	..	..	(9	11.4 3.2 to 22.4	..	(10	7.6 2.1 to 14.9	..	..	(6	4.6 1.6 to 11.6	..	..
Lambs—Older														
Cu p.p.m.	..	..	..	..	(11	* 175 83 to 309	..	(6	14.4 6.4 to 33.8	..	..	(6	‡ 18.6 9.5 to 30.0	..
Mo p.p.m.	..	..	..	..	(11	* 6.2 3.0 to 8.9	..	(6	2.4 1.7 to 3.2	..	..	(6	‡ 2.6 2.3 to 3.0	..

* = Drenched from 2 weeks old with 1/5 mother's drench = 30 mg. Mo 3 times per week.
‡ = On low copper diet from birth.

day. In 1948 and 1949, lambs which were not killed at birth were slaughtered at 3 months of age.

No ataxia has occurred in any of the lambs. Their liver copper, nevertheless, has been very markedly reduced below the Wallaceville normals of 170 p.p.m. for new-born lambs and 280 p.p.m. for lambs of 3 months of age. This effect on new-born lambs can have been due only to the molybdenum fed to the ewes. The liver copper of new-born lambs was uniformly low in the 1949 lambs, by which time the ewes had been on molybdenum treatment for nearly 4 years; but there were some low figures for lambs of 1946, when the ewes had been on this treatment for less than 1 year. The mother of a lamb born in 1946 with 13.3 p.p.m. in its liver had in her own biopsy liver sample 811 p.p.m. of copper on July 24, 1946, just 5 weeks before the lamb was born. It seems that in addition to slow and steady reduction of the liver copper of the ewe, the molybdenum drenching impeded the passage of copper to the fetal lamb's liver even before the ewe's liver copper was appreciably lowered.

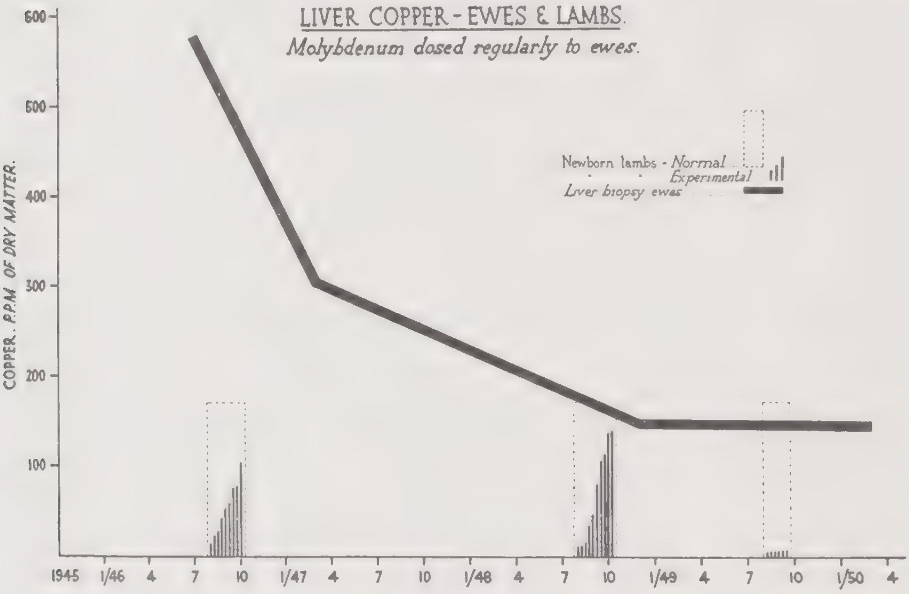


FIG. 6

The molybdenum contents of the livers of new-born lambs in this experiment were above the value of 2 p.p.m. for normal new-born lambs at Wallaceville and higher than in group mates kept until 3 months or 5 months old; higher even than group mates in 1946 which were dosed orally with molybdenum until 5 months old. Molybdenum therefore was readily transmitted through the placenta and stored in the liver of the fetal lamb. There was quite a considerable variation between individual values for molybdenum and copper of new-born lamb livers from this experiment, but no relation could be determined between the figures—for example, a high liver molybdenum in a lamb did not necessarily mean a low liver copper.

Molybdenum has been fed to sheep under a variety of conditions of copper status at the commencement of feeding and with different copper levels in the diet. Sheep grossly depleted of copper by deficiency in the diet stored copper rapidly when changed to a new diet containing 10 p.p.m. of copper. When the new diet contained in addition 30 p.p.m. of molybdenum, storage of copper was not prevented for the first 8 months, at least, but the rate of storage was reduced. Under these circumstances molybdenum was deposited in the liver and to a greater extent in the spleen and kidneys.

When 100 mg. copper was fed each day, 30 p.p.m. of molybdenum in the diet had no effect on the rate of storage of copper in the liver. Molybdenum, however, was not stored appreciably in the liver or spleen, but did accumulate in the kidneys.

Other elements, tungsten, zinc, manganese, vanadium, chromium, rhenium, uranium, or tantalum, when similarly fed to sheep on a high copper intake, did not prevent or delay the accumulation of copper in the liver.

DISCUSSION

The peat scours disease in cattle occurs always in association with a deficiency of copper shown in the soils, in the pasture fodder, and in the tissues of affected animals. The disease, moreover, is prevented or cured if extra copper is supplied to animals so that they receive a total of about 10 p.p.m. in the dry matter of the food eaten, an amount which is regarded as normal. For these reasons it is beyond question that a dietary deficiency of copper plays a deciding part in rendering cattle susceptible to peat scours.

However, a deficiency of copper in the food is not the only cause. Peat scours is restricted to definable regions in New Zealand, but there are other regions still more deficient on which cattle do not show the scouring which is characteristic of peat scours. Moreover, the deficiency of copper in pasture on peat scours land is only moderate, and does not account for the extreme depletion in affected animals. The implication is that some other factor reduces their assimilation or storage of copper.

Molybdenum fits as a second factor in the causation of peat scours. Excess dietary molybdenum is known to produce scouring (7), and scouring is a notable symptom in peat scours. This element is present in excess of normal amounts in peat scours pastures, and the content reaches a peak at seasons when scouring is most in evidence. An increased intake of molybdenum has been shown experimentally to reduce liver stores of copper in cattle, to reduce growth rate in calves, and to produce a clinical picture of scouring and unthriftiness similar to the natural cases of peat scours. The cause of this disease seems therefore to be a primary deficiency of copper, upon which is superimposed a moderate excess of molybdenum. Peat scours is in effect a molybdenosis which becomes operative when the copper status of the animal is sufficiently reduced.

A consideration of teart and peat scours and of evidence here adduced shows that the question whether molybdenum will have a pathological effect on cattle is determined by the relative amounts of copper and molybdenum in the fodder. In teart pastures a high amount of molybdenum causes scouring, despite a normal copper content, within a few days after cattle commence to eat the pasture. In peat scours pastures a moderate excess of molybdenum causes scouring only if the pasture copper is below normal and only when the body store of copper of healthy animals has been reduced by the low intake and by the depleting effect of molybdenum. For fodders of normal copper content, a higher ratio of molybdenum to copper must occur before cattle will scour. For fodders of low copper content, a lower ratio or relatively smaller amount of molybdenum is pathogenic.

The peat scours lands are but one aspect of the copper deficiency problem in New Zealand. The other occurs on regions which have been defined as simple deficiency areas. Disease of cattle on these areas can, at present, be most simply regarded as a result of a quantitative deficiency of copper. This view has been adopted, but only tentatively, since the influence that molybdenum has been shown to exert on copper metabolism suggests that any opinion on copper requirement may be subject to review as more information becomes available.

Experimental cattle on the regime associated with simple copper deficiency show a slower growth rate, a lower hemoglobin content in the blood, and a higher content of iron in the liver than similar animals to which small supplements of copper are fed. The picture is, in fact, that associated in other animal species with a deficiency of copper.

Ataxia in lambs is associated, wherever it occurs, with a deficiency of copper in the mother and in the affected lamb. In Australia (2), (3), and in many of the New Zealand cases a deficiency in the fodder accounts for the deficiency in affected animals. This is not the case in Great Britain (10) and in some of the New Zealand cases which occur on pasture that has sufficient copper to meet the requirements of sheep. In such cases the animal deficiency must be induced by some other influence which modifies the utilization of copper. Extra molybdenum has been shown to reduce the liver copper of ewes on a normal copper intake and to reduce still more the copper that is transmitted to the fetal lamb. This element may, therefore, play a part in causing ataxia in lambs on complicated deficiency areas in New Zealand. Reduced copper alone, however, does not explain the development of ataxia. The incidence of the disease varies from year to year on copper-deficient areas without a corresponding variation in the copper content of the pasture. In most affected flocks only some of the depleted lambs become ataxic, and others, equally depleted, do not develop symptoms or lesions, while experimental induction of very low copper in lambs has been achieved without any symptoms of ataxia developing. All this suggests that our knowledge of the cause of ataxia is as yet far from complete.

The work discussed in this paper has been primarily concerned with the etiology of the animal diseases described, but some information has also been obtained on the relations between copper and molybdenum in metabolism. The finding of Dick and Bull (6) that administration of molybdenum reduces liver copper in sheep and cattle has been fully confirmed and amplified. It has further been shown that copper in the diet exercises a modifying influence on liver storage of molybdenum. In fact, a reciprocal antagonism has been demonstrated between these two elements in respect to their deposition in the liver. Copper appears to be the more potent of the two, as shown by the readiness with which the adverse effects of molybdenum in peat scours pastures can be reversed by raising the copper intake, by the marked effect of very small doses on the growth of calves on the copper-deficient pastures topdressed with ammonium molybdate, and by the absence of any effect of molybdenum on the storage of copper in livers of sheep on a high copper intake. All this shows that a relationship does exist between copper and molybdenum, and it is not impossible that further work will enable this relationship to be expressed in quantitative terms.

Molybdenum readily passes the placenta of the sheep, but inhibits the passage of copper. It is stored in a higher amount in the kidney and spleen than in the liver, and storage in these organs, unlike that in the liver, is not prevented by high dietary copper. This fact may provide a useful diagnostic measure of high molybdenum intake. The high molybdenum content of the kidney is associated with a high copper content of the same organ, and high kidney copper has in turn been associated with increased copper in the circulatory fluid. The occurrence of copper in the kidney may indicate the path of excretion of copper washed out by molybdenum. Molybdenum is excreted in appreciable amounts in the urine.

A number of other elements have been examined for their effect on the storage of copper in the sheep's liver. Tungsten has been fed under conditions of low, normal, and high copper intake, and lead with normal copper intake. Neither had any effect. Rhenium has been fed with a normal copper intake; and rhenium, zinc, manganese, vanadium, chromium, uranium, and tantalum with a high copper intake. The latter technique may impose too stringent a test and may obscure some effect of the element.

So far, however, molybdenum is the only element that has been found to exert an effect on copper metabolism. This influence is paramount in inducing peat scours and may be of importance in some cases of enzootic ataxia.

SUMMARY

Copper deficiency in New Zealand occurs on peat soils and on leached soils such as coastal sands and river silts.

Diseases in cattle and sheep on some regions result from a quantitative deficiency of copper, in others from a copper deficiency which is complicated, and in part induced by an excess of molybdenum in the food.

Simple deficiency in cattle is characterized by lack of thrift, anemia, and brittleness of bones of young animals, complicated deficiency by these symptoms together with a severe seasonal diarrhea. Deficiencies of both kinds in sheep produce symptoms only in lambs, which develop osteoporosis or ataxia.

Molybdenum is regarded as the complicating factor, as it is present in the food in an amount which has been shown experimentally to account for the course of the disease.

Supplying copper, preferably by topdressing pastures annually with 5 lbs, of bluestone per acre, prevents diseases following both types of deficiency.

Molybdenum and copper are reciprocally antagonistic in animal metabolism. Copper controls the pathological effects of molybdenum and inhibits molybdenum storage in the liver; molybdenum inhibits copper deposition in the liver. Molybdenum also inhibits placental transmission of copper in the ewe.

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DISCUSSION

DR. MARSTON: Would Dr. Cunningham care to comment on the origin of the intestinal disturbance of the bovine? I think a lot might depend on the interpretation of that. Is there any evidence that the intestinal disturbance, for instance, may be alleviated by the injection of copper into the blood stream?

DR. CUNNINGHAM: There is no appreciable inflammation or local damage in the intestines of animals affected with peat scours — the scouring does not therefore result from that cause. The onset of scouring is not immediate when an animal with normal stores of copper is transferred to peat scours country. This symptom develops only when the liver copper has been depleted. The scouring is quickly relieved if copper is supplied by any route or if the affected animals are transferred to normal pastures. This would suggest that the scouring results from a local effect on the intestine which occurs, under New Zealand conditions, when copper is deficient. The local effect is presumably exerted by molybdenum.

DR. MARSTON: That is an extraordinary phenomenon: further study of it might illuminate the striking difference in reaction between sheep and cattle.

DR. DAVIS: Dr. Comar, I remember, mentioned this morning that our injected copper seemed to be much more effective and sufficient. We injected 100 mg. of copper chloride three times at 10-day intervals into an animal, and it was a full year before that animal came around with a copper deficiency again.

DR. MARSTON: Do you consider the primary cause of the scouring to be an action on the lumen of the intestine itself?

Dr. COMAR: We have found that copper seems to be secreted by the mucosa into the lumen of the intestine, and you can check that by finding a high copper concentration in the mucosa; so it may be that when you have the copper in the animal it does get secreted into the lumen, and there has an effect upon the intestinal disorder.

Dr. MARSTON: Our studies of the effect which supervenes when the intake of molybdenum by grazing sheep is increased naturally by periodic drenching with ammonium molybdate have been conducted with experimental flocks that have been subjected to two distinct grazing conditions, namely, those of our experimental field station, where normally grazing sheep do not experience the known effects of copper deficiency; and those of the copper-deficient pastures of the calcareous littoral tracts, where sheep invariably suffer the extreme consequences of copper deficiency. In the former circumstances an increased molybdenum intake equivalent to 50 mg. Mo/day has reduced, in the course of two years, the copper concentration of the liver to less than half of that of the controls; but, as yet, the animals have suffered no unknown effects. There is apparently sufficient copper still available to meet all of the physiological requirements. In the latter circumstances similar enhancement of the molybdenum intake has led undoubtedly to more rapid precipitation of the copper deficiency syndrome, but here paradoxically this treatment has maintained a higher concentration of copper in the tissues.

Dr. DAVIS: Do you feel that that is not available to the animal?

Dr. MARSTON: It appears to be so. The evidence indicates that molybdenum tends to form in the tissues a complex with copper which cannot be utilized as a source of the copper essential for metabolism. If other factors determine the role of excretion of this complex, the difference in the reaction of sheep to increased molybdenum intake under the two conditions I have mentioned might be explained. I am tempted to suggest that a phenomenon of this sort lies at the base of the different experiences of various workers in this field.

Dr. STEWARD: Might we ask to what extent these differences are associated with the fact that all these animals are living on different plants grown under different circumstances? The thing that did strike me both this morning and this afternoon is that there are seasonal differences in the content of the forage in the copper and manganese, copper and molybdenum content — that is, the difference between the New Zealand spring plants and summer plants is quite marked. There are bound to be some other differences that are seasonal too. There must be factors in the composition of those plants other than the molybdenum and the copper content which may bear on this whole picture. If we knew the metabolic environment of the plants, might that not shed considerable light on what is going on in the animal during the crisis?

Dr. Cunningham: That is very likely. In New Zealand the spring growth of pasture contains more molybdenum than the summer growth. In Somerset, England, spring pastures are higher in molybdenum and are more prone to cause "teart." Also clovers contain more molybdenum than grasses. Further, more molybdenum is taken up from soils near the alkaline side than from those on the acid side. These indicate effects of plant and environment which influence the molybdenum content of pasture and in turn the copper metabolism of animals. There must be other substances than molybdenum in plants which affect copper metabolism of animals. In Derbyshire, England, animals are deficient in copper though their fodder is not deficient and the molybdenum content is not unduly high. In certain parts of New Zealand, on river silt soils, a similar phenomenon has recently been found to occur. The plant species are the same as on other areas where no induced copper deficiency in animals occurs. The soil and environment clearly have, as Dr. Steward suggests, caused the appearance in the plant of some unknown substance which affects copper metabolism.

Dr. Marston: Rats maintained under laboratory conditions on a diet that consisted essentially in concentrated milk invariably accumulate copper in their tissues if the intake of molybdenum is relatively high. When the intake of copper is just sufficient to provide for all of their physiological functions, an additional 1 mg. Mo/day as ammonium molybdate will precipitate copper-deficiency symptoms while enhancing materially the circulation of copper in the blood, liver, spleen, kidney, and musculature of the affected animals.

Dr. Cunningham: Dr. Marston suggests that the depletion of copper in the bovine resulting from molybdenum feeding may be caused by the excretion of a complex of copper and molybdenum. This may be true also of the ovine. In this species, continued feeding of molybdenum reduces liver copper but increases kidney copper. Normal sheep's kidneys contain about 30 p.p.m. of copper, but in sheep that have been fed molybdenum for long periods the kidney copper content goes up to about 100 p.p.m., and molybdenum in the kidkney increases from a normal of 2-3 p.p.m. to 25 or 30 p.p.m. The simultaneous excretion of copper and molybdenum via the kidney might explain the increases in the amounts of these elements present.

Dr. Marston: Sheep of different strains differ markedly in their capacity to excrete abnormal substances: for instance, during the hemolytic crises associated with chronic copper poisoning, British breeds of sheep almost invariably succumb through failure of the kidney to excrete the hemoglobin. Merino sheep, however, are better able to deal with the hemoglobin and so are able to survive crises of this sort. In the light of this experimentally established fact it would not be surprising if the ovine and bovine reactions toward copper differed; but it would be interesting to know the underlying reason for this difference. We have here a phenomenon which with little doubt is of great importance to our understanding of the maladies associated with copper deficiency and copper excess.

Dr. CUNNINGHAM: There is a very marked difference between the bovine and the ovine in the capacity to excrete copper. In the case of the bovine one can feed 5 g. copper sulfate per day for a period of at least 18 months without causing any toxicity. In the case of the sheep 1½ to 2 g. per day is fatal for British breeds within 30 days.

COPPER METABOLISM IN HUMAN SUBJECTS

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INTRODUCTION

Copper as a remedy for various ailments in man has been used since the time of the Egyptians, 1500 years ago. The Greeks in the time of Hippocrates prescribed copper compounds for pulmonary diseases. In the Western world, Paracelsus recommended copper for the treatment of mental diseases, lues, and afflictions of the lungs, so that by the eighteenth century copper compounds had come into wide clinical use. During the nineteenth century the use of these compound began to decline, even though about 1875 (54) copper was recognized as a normal constituent of blood. The true importance of this element in the nutrition of mammals was not appreciated, however, until 1924, at which time those studies were begun at the University of Wisconsin by Hart, Steenbock, Elvehjem, and coworkers (19) which demonstrated that rabbits and rats, raised on a diet consisting wholly of milk, develop an anemia which fails to respond to iron but does respond to the pale blue copper-containing ash of liver. This discovery led to a renewal of interest on the part of clinicians, especially pediatricians and hematologists, and in the early nineteen-thirties many conflicting reports appeared concerning the value of copper in the treatment of microcytic hypochromic anemia (8). Although considerable evidence was presented that in the nutritional anemia of infants the rate of hemoglobin formation is accelerated when copper is given in addition to iron, the general opinion emerged that most patients with microcytic hypochromic anemia respond satisfactorily, rapidly, and maximally to iron medication alone. As a consequence, the interest of clinicians in copper again waned, work in the field decreased, and the importance of copper in the metabolism of the human body received little attention. This is extremely unfortunate, since the observation that clinical anemias fail to respond to copper in no way proves that copper is not essential for hemopoiesis or that this element is not an essential or important constituent of the diet. The clinical studies simply demonstrated that the quantities of copper needed are so small that under the conditions of clinical experimentation sufficient copper is present in the body stores, in the diet, and as a contaminant in the therapeutic iron to supply the needs.

It is the purpose of this paper to summarize present knowledge concerning the metabolism of copper in human subjects and to present evidence that in many pathological conditions the metabolism of this element is greatly altered. Unfortunately, our knowledge is so limited that interpretations of the functions and significance of these alterations are not possible at this time. Since the newer advances in the study of copper metabolism in animals are being discussed by others in this symposium volume, this account will be concerned mainly with human subjects.

The original work * reported in this paper has been carried out in the Department of Medicine at the University of Utah in conjunction with Dr. M. M. Wintrobe, Dr. Clark Gubler, and Dr. Eugene Lahey.**

ABSORPTION AND EXCRETION

Very few studies have made on the absorption of copper in man. Information is not available as to whether organically bound copper is absorbed as well as copper in inorganic form. In animals it has been shown that the copper of wheat germ, alfalfa, brewer's yeast, pork heart and liver, copper glutamate, copper glycerophosphate, cysteine cuprous mercaptide, copper aspartate, copper citrate, copper tyrosine, copper nucleinate, and copper pyrophosphate is readily utilized by severely anemic rats (65). Copper sulfide and copper porphyrin are not utilized by animals, whereas the oxide, hydroxide, and iodide are readily utilized. There are also no data as to whether in man the cupric or the cuprous form is more readily absorbed. Studies concerning the effects of gastric acidity, the motility of the bowel, and the constitution of the diet on the absorption of copper have not yet been carried out. Likewise, there are no data as to which part or parts of the gastro-intestinal tract are active in the absorption of this element. Studies in dogs (60) indicate that copper is absorbed in the upper jejunal loops but not at all in the middle and distal loops. Considering the great amount of work which has been done regarding the factors which influence the absorption of iron in human subjects, it is strange that so little has been done on this phase of copper metabolism.

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** National Research Council Fellow.

Van Ravesteyn (71) administered 200 mg. of copper sulfate by mouth to 3 normal human subjects and failed to observe a rise in level in the plasma. From this he concluded that following absorption copper is immediately deposited in the liver. Schubert and Riezler (62), using radioactive copper, were able to detect only small quantities in the blood after oral administration. However, studies in animals, shown in Fig. 1, indicate that if large doses of copper are given there is a considerable rise in plasma copper. Similar results have been obtained in dogs by others (59, 72).

Copper is excreted into the intestinal tract and through the kidneys, and is lost in the normal menstrual flow and in sweat. The chief channel of excretion is apparently via the intestinal tract. Van Ravesteyn (71) found the normal copper content of the bile varies from 35 to 205 μg. per cent. Following the intravenous injection of copper, he noted a temporary rise in the copper content of the bile in 2 to 4 hours. After a few days there was a secondary rise in biliary copper and a significant increase in the copper content of the feces. The fact that the excretion of copper in the bile and via the feces did not appear to run parallel led Van Ravesteyn to conclude that at least part of the copper found in the feces is excreted directly through the intestinal wall. After 3 injections of a total of 33 mg., the excretion of this element in the stools increased 2.5 mg. per day, so that within a few weeks most of the administered copper had been excreted into the intestinal tract. Similar results

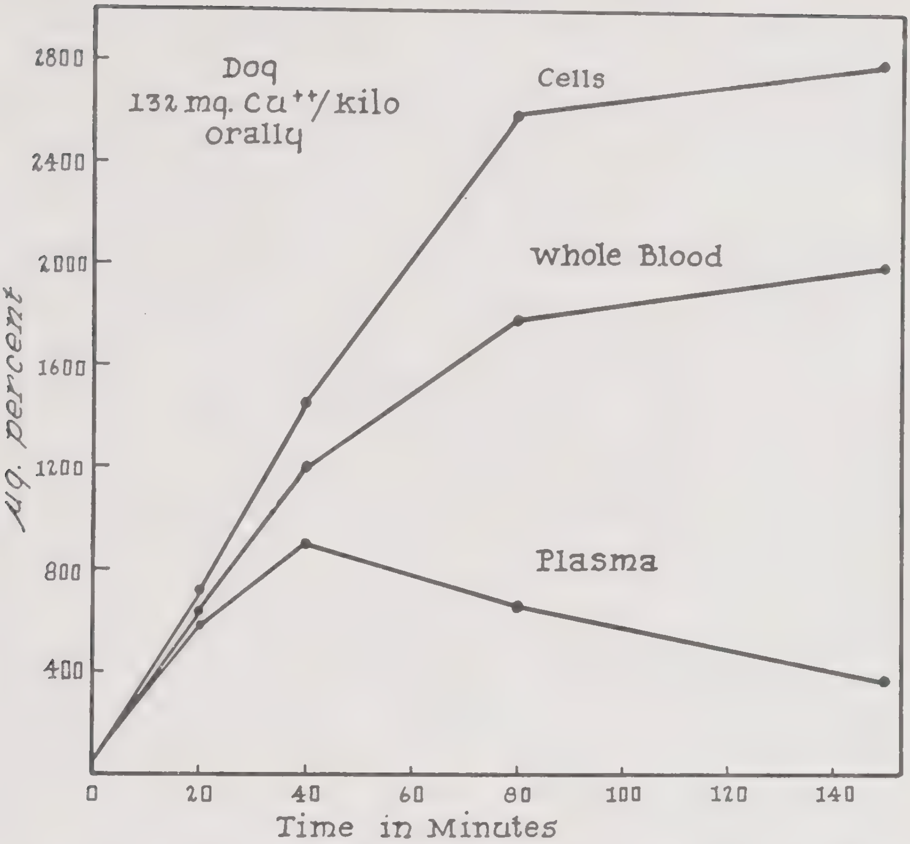

FIG. 1. Increase in plasma, red cell, and whole blood in a dog following a single oral dose of 0.66 g. of $CuSO_4$ per kilogram of body weight, given at zero time.

TABLE 1

EXCRETION OF COPPER IN THE URINE OF NORMAL HUMAN ADULTS

Investigator	No. of Subjects	Copper Intake mg./24 hrs.	Urinary Copper mg./24 hrs.	
			Mean	Range
Rabinowitch (56)	50	....	0.29	0–0.70
Tompsett (69)	24	2.40	0.28	0.12–0.52
Chou and Adolph (14)	4	2.31	0.26	0.15–0.36
Holt and Scoular (33)	17	8.10	0.21	0.02–0.67
Van Ravesteyn (71)	4	....	0.03	0–0.10
Leverton (35)	24	2.50	0.20	0.03–0.32

were obtained by Schubert, Maurer, and Riezler (63) in man, using Cu^{64}.

The normal copper content of the urine as determined by various authors has been tabulated, and the data are presented in Table 1. The normal excretion of copper through the kidneys in adults is about 0.25 mg. per 24 hours. The excretion in children is proportionately less. There is apparently no correlation between the amount of copper ingested and the amount excreted in the urine over the range of 1 to 11 mg. of copper per day. Likewise, Van Ravesteyn (71) was unable to demonstrate an increase in the excretion of copper in the urine after the administration of copper sulfate by mouth. Following the intravenous administration of copper there was only a slight, transient increase in the urine. From this it may be concluded that the kidneys are not an important route for the excretion of copper.

Copper loss through normal menstrual flow is not great. Leverton and Binkley (36) found that the average loss per period for each of four subjects was 0.32 mg., 0.48 mg., 0.65 mg., and 0.74, with an average of 0.55 for all the subjects.

A small insignificant amount of copper is lost in sweat. Mitchell and Hamilton (45) found that sweat contains approximately 5.8 μg. of copper per 100 ml.

STORAGE

It has been estimated that the adult body contains between 100 and 150 mg. of copper (14). The muscle mass contains a total of about 64 mg., the bones 23 mg., and the liver 18 mg. The remainder of the copper is distributed rather evenly throughout the body. The concentration of copper in the various tissues has been given as follows (mg. per kilogram of dry tissue): liver, 24 to 60; kidney, 17 to 48; heart, 10 to 13; brain, 23 to 50; pancreas, 4 to 28; spleen, 5 to 20; lungs, 17; hair, 15; bone, 6 (1, 3, 16, 26, 70). The concentration of copper in the liver is higher than in other organs, the values being about 7 μg. per gram of wet tissue (1). The copper concentration in fetal liver is 5 to 10 times that of adult liver (1, 47). During fetal life the concentration increases to a maximum at term and then during the first year of life decreases steadily, but at one year it is still higher than in adult liver (1, 3, 47). Between the ages of 1 to 5 years the level remains high, and it is not until the age of 5 to 15 years that values equal to those of adults are obtained (1).

The copper content of the liver has been noted to decrease in nutritional anemia (14, 47) and is increased in Mediterranean anemia (26, 61), hemochromatosis (42), cirrhosis of the liver (42), acute yellow atrophy of the liver (22), tuberculosis (3), carcinoma (4, 24, 61), and severe chronic diseases accompanied by anemia (61). The increase in copper in the depot organs is marked in patients with malignant disease and is highest in those with metastases.

TABLE 2

Copper Balance in Normal Human Subjects

Investigator	No. of Subjects	Type of Subjects	Copper Intake mg./day	Copper Excretion mg./day	Balance mg./day	Per cent Retention
Tompsett (69)	6	Adults	2.40	2.37	+0.03	1.2
Chou and Adolph (14)	4	Adults	2.31	1.97	+0.34	14.7
Ohlsen and Daum (53)	3	Adult Females	1.10	1.17	−0.07	0.0
Leverton (35)	24	Adult Females	2.50	2.00	+0.50	20.0
Leverton and Binkley (36)	65	Adult Females	2.65	1.80	+0.85	32.0
Holt and Scoular (33)	17	Adult Females	8.10	0.05	+7.6	93.8
Scoular (64)	3	Male Children	1.58	0.61	+0.97	61.4
Daniels and Wright (17)	6	Children	1.59	1.17	+0.42	26.4
Macy (40)	7	Children	5.03	2.02	+3.01	59.8

DIETARY REQUIREMENT

By careful balance studies it has been possible to determine the dietary copper requirement of man. Quite a few studies have been carried out, and these are summarized in Table 2. In the adult, positive copper balance is maintained if the dietary intake is approximately 2 mg. per day, or greater. This is illustrated in Fig. 2. Most workers agree that if the intake falls below about 2 mg. per day, patients develop a negative balance. It is also apparent that as the copper intake increases there is an increase in the amount retained, up to an intake of about 8 mg. per day. Beyond this dietary level there is no significant increase in the amount of copper retained.

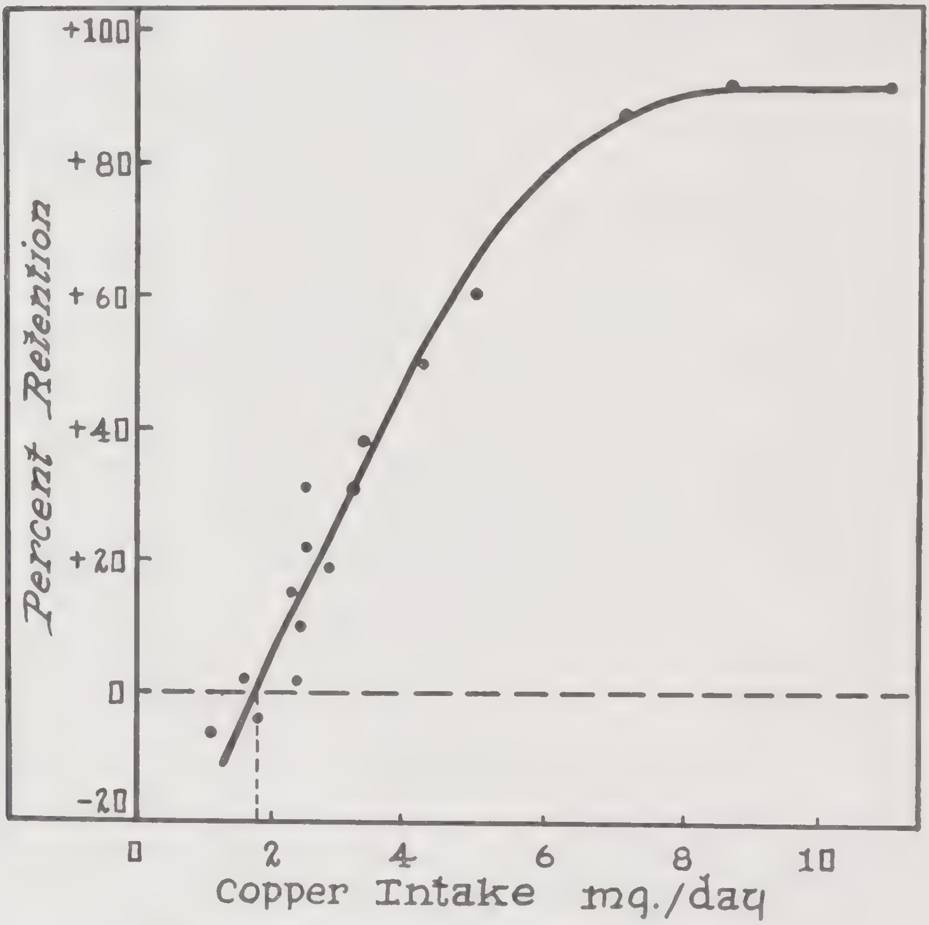

FIG. 2. Copper balance in normal human subjects. Data from the literature (see Table 2).

Since copper loss through menstrual flow is not great, no difference has been detected between the copper requirement of males and non-pregnant females. The dietary requirement of pregnant women is of course increased, since fetal stores of copper are high.

The exact dietary requirement for pregnant women has not been determined.

The dietary copper requirement for children, from the limited data available (Table 2), would seem to be about 80 μg. per kilogram of body weight. Scoular (64) found the daily copper requirements of boys 3 to 6 years of age to be 53 to 85 μg. per kilogram. Macy (41) has stated that for children 8 years of age the requirement is 100 μg. per kg., and for children 11 years of age about 80 μg. per kg. of body weight.

An American diet of even mediocre quality easily supplies the daily requirement. Leverton and Binkley (36) found that young women on a self selection diet ingest about 2.5 mg. of copper per day. According to Macy (41), an 8-year-old child eating an average diet receives about 4.9 mg. of copper per day, and a child of 11 years receives about 5.2 mg. Hodges and Peterson (28) have computed that the average American family consumes about 2.26 mg. of copper per day per person.

The copper content of foodstuffs varies considerably, depending upon the copper content of the soil from which the foods have been obtained. Lindow, Elvehjem, and Peterson (37) have classified foods in descending order of their copper content as follows: nuts, dried legumes, cereals, dried fruits, poultry, fish, animal tissues, green legumes, roots, leafy vegetables, fresh fruits, and non-leafy vegetables. Cow's milk is relatively low in copper (1). During the spring and summer it contains 0.17 to 0.50 mg. per liter and during the winter 0.09 to 0.21 mg. per liter. The copper content increases during transport in metal vessels and during pasteurization in metal containers. The copper content of breast milk is somewhat higher than that of cow's milk. Colostrum contains 0.95 to 1.23 mg. per liter, and breast milk in the second month contains 0.60 to 0.95 mg. per liter. The copper in breast milk decreases with nursing, so that in the 9th month it contains about 0.30 to 0.70 mg. per liter.

Blood Copper in Normal Individuals

Earlier investigations of the copper content of blood were limited because the methods available at the time were cumbersome and inaccurate. Consequently there was considerable disagreement as to the amount of copper in blood. In recent years simple and reliable methods have been developed for the determination of copper in body fluids, and, as a result of the work of such investigators as Heilmeyer (27), Holmberg (29), Nielsen (50), Sachs (58, 60), Munch-Petersen (49), Axtrup (1), and our own group (11, 25), reliable data concerning values for whole blood, red cell, and plasma copper in both normal individuals and in patients with disease are available. The earlier literature has been reviewed by Axtrup (1), Heilmeyer (27), and Cartwright (8).

TABLE 3

Normal Values for Whole Blood Copper

Investigator	Year	Sex	No. of Individuals	Mean μg. %	±S. D.	Range μg. %
Sachs et al. (60)	1943	Male Female	10 10	102 107	± 5.9 ± 6.0	90–109 101–121
Gubler et al. (25)	1950	Male Female	12 11	91 96	± 9 ±12	70–107 69–119
Sarata (1)	1934	Mixed	10	99	±13	91–108
Holmberg (29)	1941	Female	22	97	±16	73–122
Heilmeyer et al. (27)	1941	Mixed	8	110	±19	87–147

Adults. Whole blood contains about 94 ± 10 μg. of copper per 100 ml. Values obtained by various workers are summarized in Table 3. The individual variation is from 70 to 120 μg. per cent.

There is now excellent agreement among investigators as to the normal values for plasma copper (Table 4). The mean values given by 7 different groups of investigators range only from 92 to 123 μg. per cent, with a range of individual determinations from 64 to 165.

The copper in blood is distributed approximately equally between cells and plasma. In our laboratory, using a recently developed highly accurate method (25), we have found the copper content of the erythrocytes to be 74.3 ± 4.94 μg. per 100 ml. in males and

76.6 ± 2.75 μg. per 100 ml. in females. This gives a ratio of cell copper to plasma copper of 0.65 ± 0.041 in males and 0.75 ± 0.051 in females. This is somewhat lower than the quotient obtained by Heilmeyer, Keiderling, and Stuwe (27). We have been able to calculate that the amount of copper in the average red corpuscle (mean corpuscular copper) is about 65 ± 10.8 μ μ μg., and that the concentration of copper in the red cell is normally about 0.000075 per cent. The copper content of leukocytes and platelets is considerably less than in erythrocytes, the value being about 20 μg. per 100 ml. Since the ratio of the volume of erythrocytes to leukocytes and platelets in normal blood is approximately 47/0.7, failure to separate the leukocytes and platelets from the erythrocytes results in an insignificant error.

TABLE 4

NORMAL VALUES FOR PLASMA COPPER

Investigator	Year	Sex	No. of Individuals	Mean μg. %	S. D.	Range μg. %
Nielsen (50)	1944	Male	30	110	±12	75–165
		Female	100	123	±16	75–165
Heilmeyer et al. (27)	1941	Male	30	107	±16	80–138
		Female	30	106	±17	72–134
Cartwright et al. (11)	1948	Male	52	114	±15	86–161
		Female	53	123	±11	87–161
Gubler et al. (25)	1950	Male	12	105	±17	68–126
		Female	11	114	±15	81–141
Robinson (57)	1949	Male	10	92	±19	64–112
		Female	8	104	±16	79–125
Munch-Petersen (49)	1948	Male	50	108	±15	81–164
		Female	50	118	±16	73–152
Holmberg (29)	1941	Female	22	110	±24	70–149

In our laboratory it has been found that there is no significant difference in whole blood, plasma, or red cell copper between males and females (Table 5). With this conclusion all investigators are in agreement except Nielsen (50), who found that women have a statistically significantly higher plasma copper than men. The values for normal whole blood copper, plasma copper, cell copper and the various copper indices, obtained in our laboratory by improved methods described elsewhere (25), are presented in Table 5.

Infants and Children. There are few studies of the blood copper content of infants and children as measured by newer methods.

TABLE 5

Blood Copper Values in Normal Individuals

Sex	No. of Subjects	Whole Blood Copper μg./100 ml.	Plasma Copper μg./100 ml.	Cell Copper μg./100 ml.	Cell Copper / Plasma Copper	Mean Corpuscular Copper μ μ μg.
Male	12	91.5 ±2.62	105.5 ±5.03	76.6 ±2.75	0.75 ±0.051	66 ±7.9
Female	11	96.5 ±3.54	114.0 ±4.67	74.3 ±4.49	0.65 ±0.041	64 ±4.2
Total	23	93.9 ±2.20	109.5 ±3.50	75.4 ±2.71	0.70 ±0.033	65 ±2.2

Figures represent mean ± standard error.

Axtrup (1) has made the most comprehensive study and reviewed the literature thoroughly. The plasma copper of 5 to 6 month old fetuses is high, ranging in the region of 195 to 235 μg. per cent. The plasma of newborn infants is low as determined in umbilical vein blood, the mean value of various investigators varying from 53 to 83 μg. per cent. In our own laboratory (20) the serum copper determined from the umbilical vein in 14 newborn infants was found to be 75 ± 14 μg. per cent, while the maternal serum copper was 260 ± 42 μg. per cent. During the first ten days of life the copper in the plasma and red cells increases. By the second week of life the values reach the adult levels and remain at this level throughout childhood.

Variations in Plasma. The plasma copper values vary relatively little from individual to individual. In a total of 128 normal individuals of both sexes a range of 68 to 161 μg. per cent was found. In the same individual the values for whole blood as well as plasma remain remarkably constant from day to day and week to week (Fig. 3). The fluctuation is less than ± 30 per cent. We have been unable to observe a diurnal variation in plasma copper (Fig. 3), nor has Heilmeyer (27). Neilsen (50), on the other hand, found the values to be lowest in the morning, rising somewhat during the day, and attaining the highest values in the evening. No tendency for an increase in the plasma copper following meals, or for a decrease during fasting has been observed. Physical exertion, according to Heilmeyer, apparently has no effect. Variations during the menstrual cycle have been found to be within the physiological variation of ± 30 per cent (25, 27, 50). No difference has been noted between the copper content of plasma and serum.

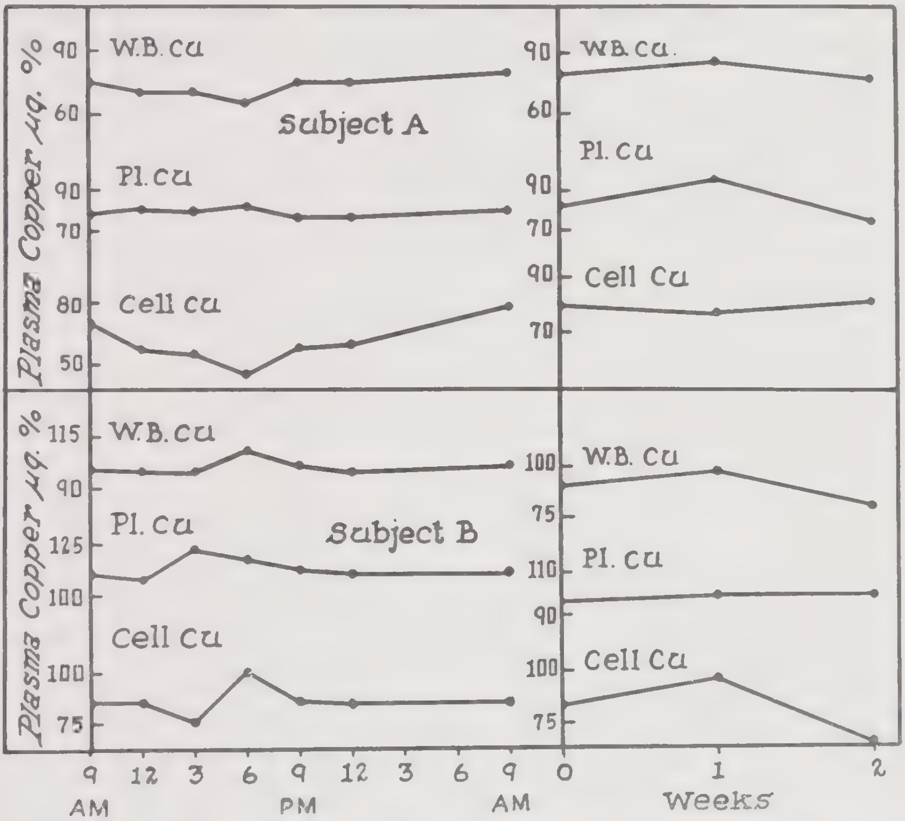

FIG. 3. Variation in whole blood, plasma, and red cell copper in two normal subjects during the course of a day, and from week to week.

The factors which normally regulate the plasma copper level have been studied very little. In our own laboratory (12) it has been found that the factors which are so important in the regulation of the plasma iron level, such as dietary intake, tissue stores, the functional activity of the adrenal cortex, and the functional state of the reticulo-endothelial system, play little or no part in the regulation of the plasma copper level under normal circumstances. Nielsen (52), as well as certain earlier investigators, observed a correlation between serum copper and the basal metabolic rate in patients with hyper- or hypo-thyroidism. The correlation was poor, however, and the values were within the normal range except in several patients with severe thyrotoxicosis. The exact role of the thyroid gland in the regulation of the plasma copper level needs to be studied.

Copper Protein of Plasma. Holmberg and Laurell (30, 31) have isolated a blue protein in a relatively pure state from the serum of man. This protein contains 90 per cent, if not all, of the copper in the serum. They have named the new protein *caeruloplasmin* and identify it as an α_2 globulin. Caeruloplasmin has been found to have a molecular weight of about 151,000 and contains 8 atoms of copper. It can be reduced to a colorless compound with certain reducing agents, and this reduction is completely reversible in the presence of oxygen. The relation of this copper protein to the copper protein, hemocuprein, described by Mann and Keilin (44), is not entirely clear. However, since hemocuprein contains 2 atoms of copper in a molecule weighing about 35,000, Holmberg and Laurell suggest that their protein may contain four units of hemocuprein.

The function of caeruloplasmin is not known. Holmberg and Laurell (32) observed that when a purified preparation of this copper globulin is added to normal human plasma, a marked increase in the histaminolytic activity of the plasma takes place. On the basis of this they suggest that the blue globulin may be a component of the histaminolytic enzyme system. This observation needs confirmation.

It would seem unlikely that this compound serves the purpose of transporting copper absorbed from the gastro-intestinal tract since, unlike the β_1 globulin which transports iron, it is entirely bound to copper under normal circumstances, and none is free to take up additional amounts of copper. Furthermore, after the oral or intravenous administration of a relatively large quantity of copper, it can be demonstrated that this additional copper in the plasma reacts directly with diethyldithiocarbamate even though it is in the main non-dialyzable, whereas the original plasma copper, the caeruloplasmin, does not (Fig. 4). This would suggest that only a negligible quantity of the copper normally present in plasma represents copper in transport and that copper, while being transported, is loosely bound to other proteins. It is well known, for example, that albumins are capable of loosely binding copper in vitro.

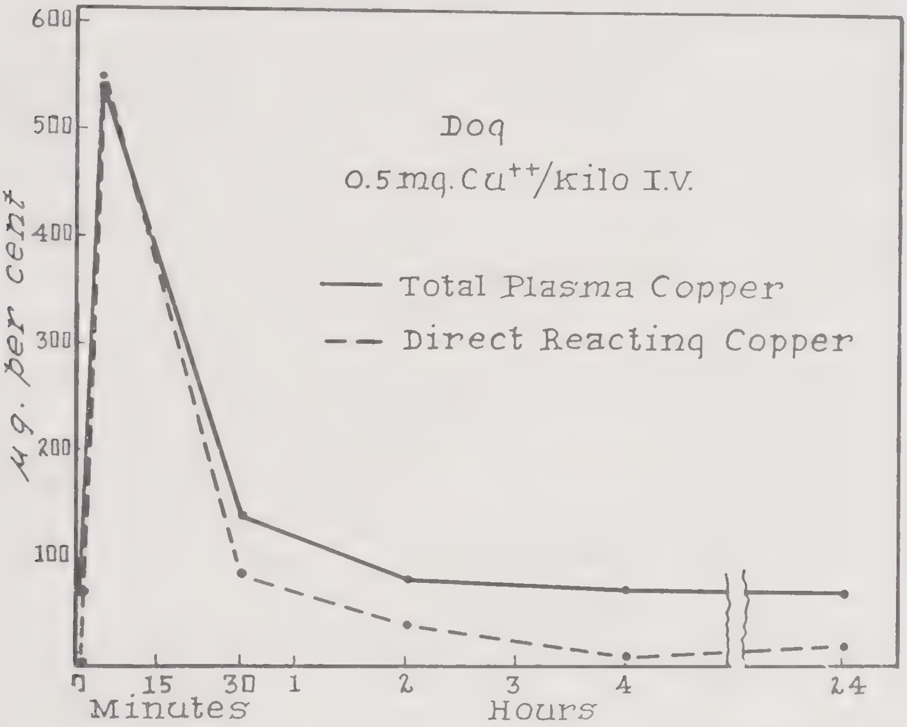

FIG. 4. The copper normally present in plasma does not react directly with diethyldithiocarbamate. Following intravenous injection of copper, there is an increase in total plasma copper which is due entirely to the increase in direct-reacting copper.

Cohn (15) has shown that the iron-binding protein, β_1 globulin, Fraction IV-7, is capable of uniting reversibly with copper. Although this protein preferentially binds iron, and the addition of copper to serum does not affect its iron-binding capacity, it is still possible that this protein functions as a transport protein for copper.

Studies on the copper content of human sera in relation to the serum protein fractions, as determined by electrophoretic analysis, reveals little or no correlation between copper and total protein, albumin, β globulin, and γ globulin. There is, however, a good correlation between the $\alpha_2 + \alpha_3$ globulin fraction and the serum copper concentration, as shown in Fig. 5. There is an even better correlation between α_2 globulin and copper, the correlation coefficient being + 0.84.

TRANSFER OF COPPER FROM SERUM TO RED CELLS

It was shown in Fig. 1 that following the oral administration of a large quantity of copper there is an appreciable increase in the amount in the plasma within 20 minutes. There is also a marked increase in the amount in the red cells. This would seem to indicate that there is rapid transfer of this element from the plasma into or onto the red cells. After about 40 minutes the plasma copper begins to fall, but at this time the copper of the red cells is still increasing. A similar transfer of copper from the plasma to the cells can easily be demonstrated in vitro.

In Fig. 1 it is further shown that the red cell copper increased in a dog to the remarkably high level of about 2.8 mg. per 100 ml. of red cells following a single oral dose. Such copper cannot be removed from the cells by repeated washings with saline. Whether the copper is present in the form of hemocuprein, is now under investigation. The number and nature of copper compounds in red cells are not known, nor is there any information as to the function of red cell copper. Mann and Keilin were unable to demonstrate any catalytic activity by hemocuprein.

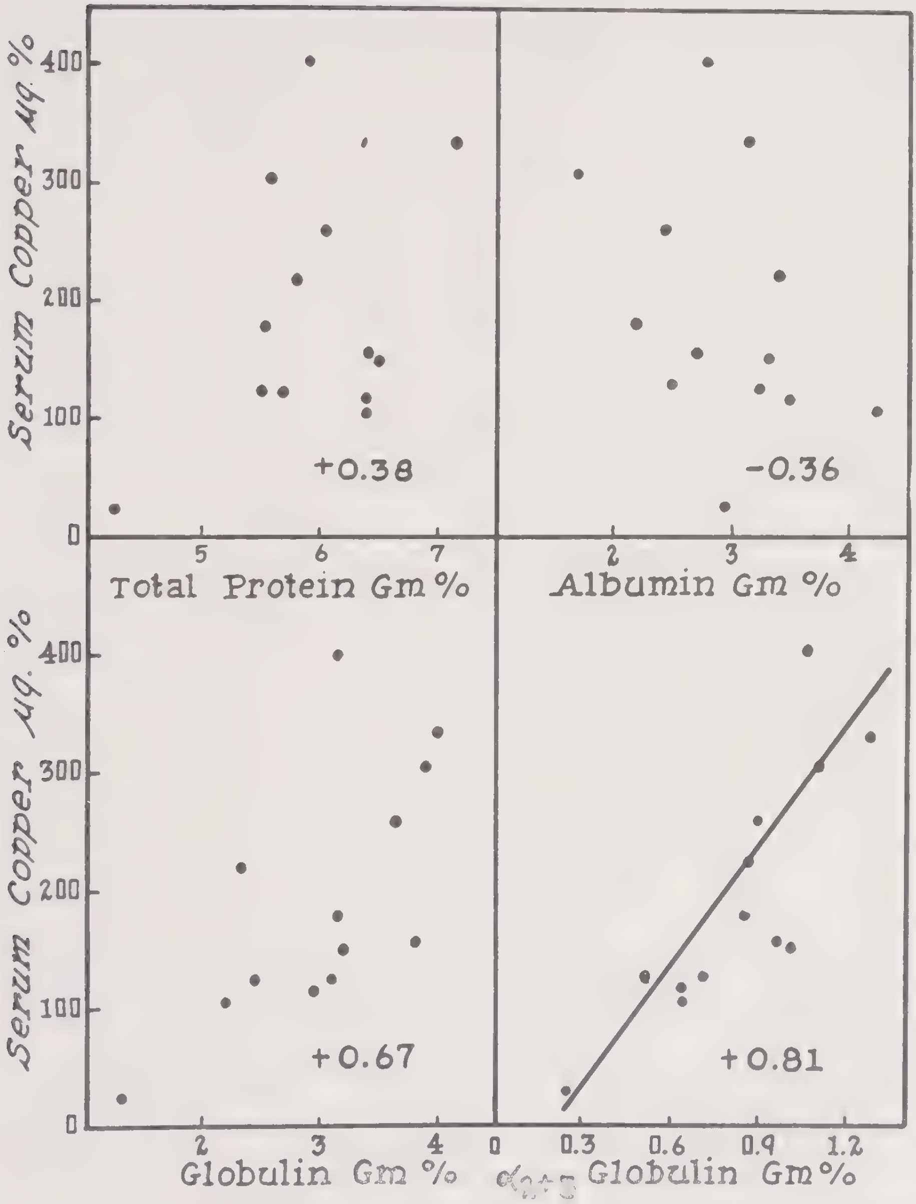

FIG. 5. Correlation of the serum copper level with total protein, albumin, globulin, and $\alpha_2 + \alpha_3$ globulin, as determined by electrophoresis. The values given are those of the correlation coefficients.

Blood Copper in Various Clinical Conditions

Infections. In 1928, Krebs (34) discovered that the serum copper was increased in acute as well as in chronic infectious diseases. His results have since been verified by a number of investigators, including Heilmeyer et al. (27), Gorter et al. (23), Cartwright et al. (7, 11), Munch-Petersen (49), and Robinson (57).

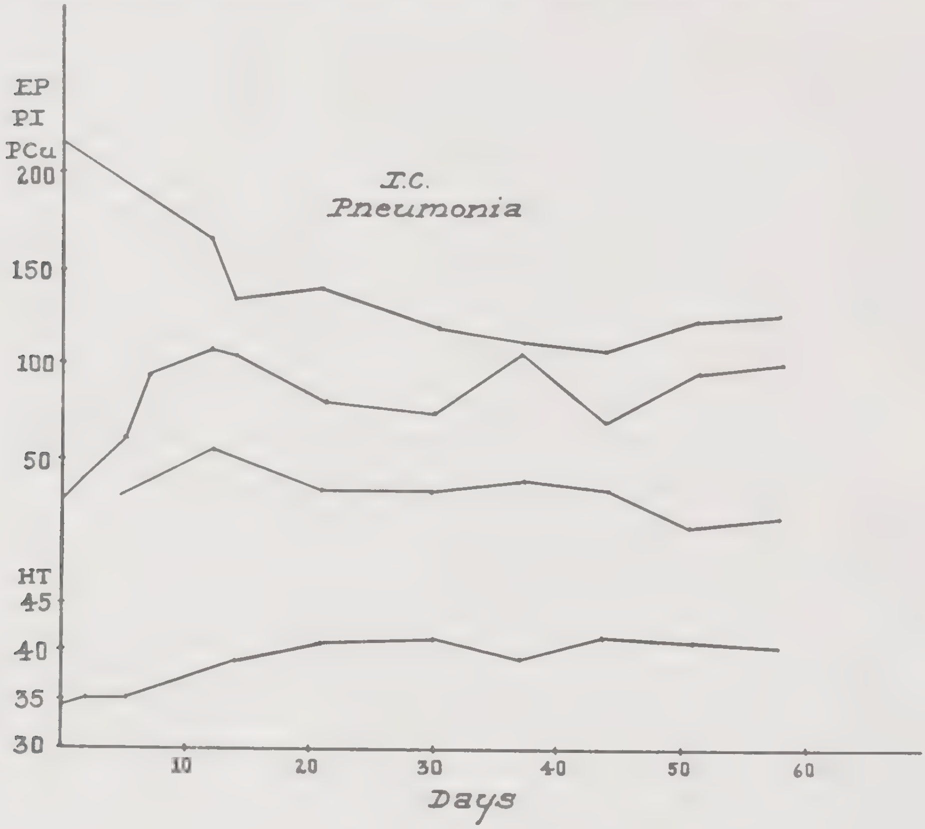

Fig. 6. Chemical changes in the blood of a patient with lobar pneumonia and early lung abscess with anemia, hypoferremia, and hypercupremia. Following therapy with penicillin, as the anemia disappeared, the plasma iron rose and the plasma copper decreased to normal.

P Cu, plasma copper expressed in μg. per cent; PI, plasma iron expressed in μg. per cent; EP, free erythrocyte protoporphyrin expressed in μg. per 100 ml. of red cells; Ht, volume of packed red cells expressed in ml./100 ml. (Cartwright, et al., *Blood,* 3: 501. 1948).

The copper in the plasma increases in most infections accompanied by fever and an increased sedimentation rate. The rise occurs soon after the onset of the infection. It occurs even with mild infections, such as pharyngitis (49) and upper respiratory tract infections, even in the common cold (27). The presence of hypercupremia has been suggested as a delicate test for the detection of infection. As soon as the acute phase of the infection subsides, the plasma copper decreases to normal.

TABLE 6

BLOOD COPPER IN VARIOUS DISEASES

Condition	No. Patients	Whole Blood Copper μg. %	Plasma Copper μg. %	Cell Copper μg. %	Ratio Cell Cu / Plasma Cu	M. C. Cu μμμg.
Normal	23	94 ±2.2	109 ±3.5	75 ±2.7	0.70 ±0.033	65 ±2.2
Chronic Infection	18	137 ±5.0	163 ±7.7	100 ±5.1	0.64 ±0.004	86 ±0.4
Pregnancy	11	163 ±7.6	220 ±13.2	68 ±10.5	0.33 ±0.06	58 ±9.2
Acute Leukemia	4	206 ±41.7	227 ±48.8	151 ±25.4	0.70 ±0.09	135 ±18.0
Chronic Leukemia	4	110 ±9.6	128 ±9.2	86 ±17.3	0.67 ±0.13	74 ±14.0
Hodgkins Disease	3	127 ±16.0	160 ±21.0	76 ±4.4	0.49 ±0.06	64 ±3.2
Iron Deficiency	6	118 ±6.9	128 ±9.0	106 ±19.7	0.90 ±0.17	80 ±11.8
Pernicious Anemia	5	123 ±9.4	134 ±7.3	87 ±20.0	0.64 ±0.12	100 ±25.0

Values are means ± standard errors.
M. C. Cu refers to mean corpuscular copper.

In chronic infections such as tuberculosis, osteomyelitis, empyema, lung abscess, subacute bacterial endocarditis, and brucellosis either with or without anemia, the plasma copper is almost invariably elevated to levels of 160 to 250 μg. per cent. The fall in plasma copper during the recovery of a patient suffering from lobar pneumonia is shown in Fig. 6. In such conditions as active rheumatic fever, active rheumatoid arthritis, and lupus erythematosus dissiminatus there is also an increased plasma copper level.

As summarized in Table 6, in infections there is an increase in whole blood and cell copper as well as in plasma copper. The average amount of copper in a red cell is increased from 65 to 86 $\mu\mu\mu$g. The increase in red cell copper is proportional to the increase in plasma copper, so that the ratio of cell copper to plasma copper is essentially unaltered. These results are somewhat in disagreement with those of Heilmeyer (27), who found a reduction in the content of the erythrocytes and postulated that in infectious diseases there was a shift in the copper from the cells to the plasma.

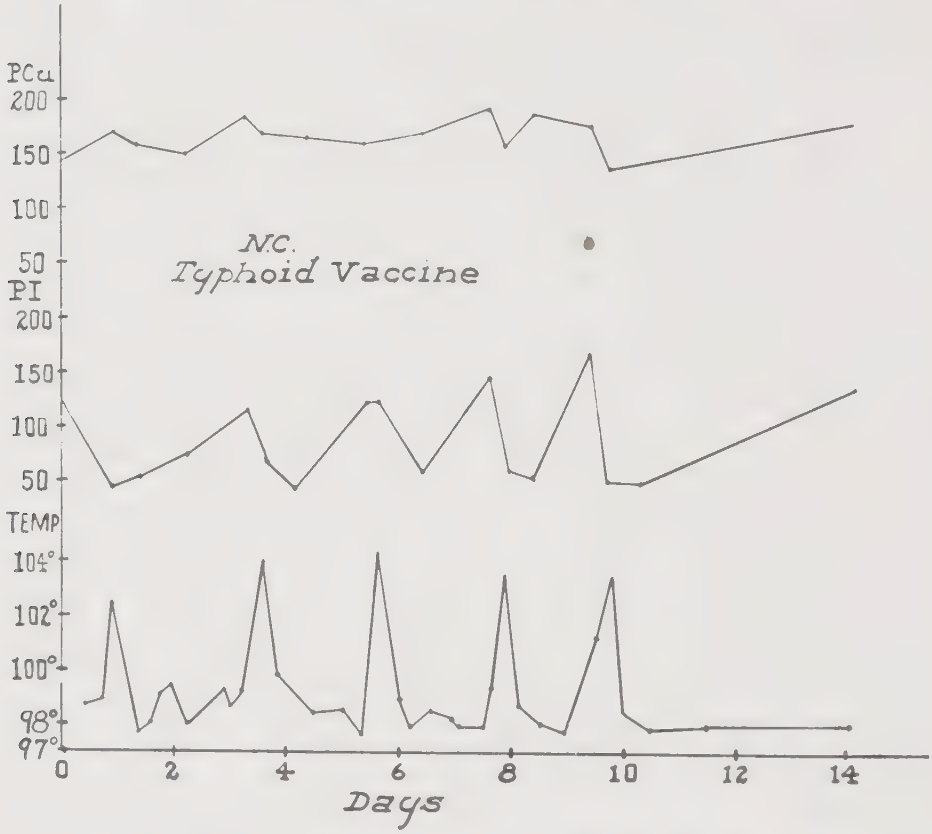

FIG. 7. The effect of induced fever (typhoid vaccine) on the plasma copper (P Cu) and plasma iron (PI). With each paroxysm the plasma iron decreased markedly and returned to normal shortly after the cessation of fever. There was no significant change in the plasma copper. (Cartwright et al., *Blood,* 3: 501. 1948).

Heilmeyer (27), in experiments in man, has found that agents which activate the reticulo-endothelial system, such as injections of milk, typhoid vaccine, and diphtheria antitoxin, cause a rise in the serum copper. In our laboratory it has not been possible to confirm this finding, a fact illustrated in Figs. 7, 8, and 9. In animals following the injection of turpentine or following the administration of colloidal thorium dioxide, which is a reticulo-endothelial " blocking " agent, we have been unable to detect any alteration in the plasma copper levels. These studies are preliminary and are now being extended.

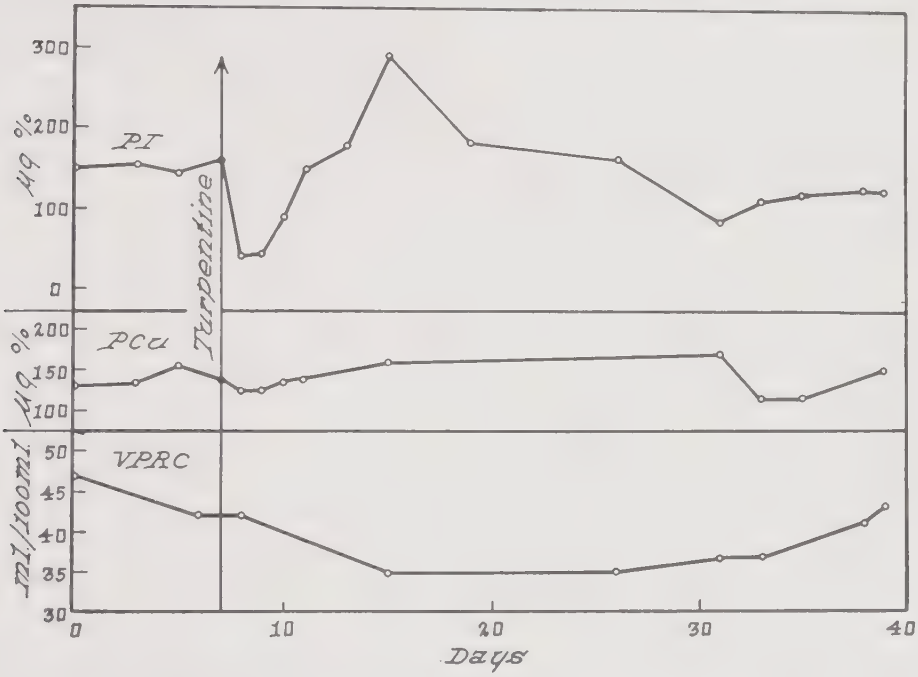

Fig. 8. The effects of intramuscular injection of 5 ml. of turpentine on the plasma iron (PI), plasma copper (PCu), and volume of packed red cells (VPRC) of a dog. An acute hypoferremia resulted but the plasma copper level was unaffected. (Cartwright, Gubler and Wintrobe, *J. biol. Chem.*, 184: 579. 1950).

Why there is an increase in red cell and plasma copper in infections, where the copper is mobilized from and what function or functions it serves, are not known. Since the increase in plasma copper occurs in acute infections without any obvious alteration in erythropoiesis occurring, it is possible and even likely that this alteration is unrelated to hemopoiesis. It might be, although this is entirely speculative, that the copper is related to the antigen-antibody system and functions in some way in the defense mechanism of the body; or it could be that the altered copper metabolism is simply a manifestation of the increased metabolic rate of the cells. This phase of copper metabolism is extremely important and deserves intensive study.

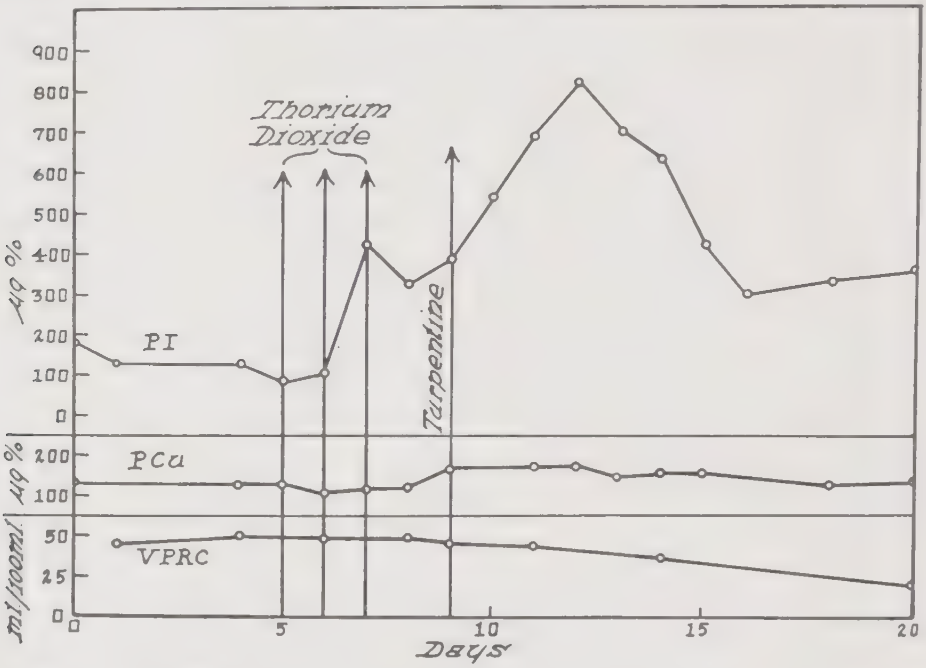

FIG. 9. The effects of three intravenous injections of colloidal thorium dioxide (2 ml. per kg. of body weight) on the plasma iron (PI), plasma copper (PCu) and volume of packed red cells (VPRC) of a dog. Although the thorium produced a marked hyperferremia, the plasma copper level remained constant. Following thorium, turpentine had no effect on either the plasma iron or copper level. (Cartwright, Gubler and Wintrobe, *J. biol. Chem.,* 184: 579. 1950).

Pregnancy. An increase in the plasma copper in pregnancy was first noted in 1928 by Krebs (34) and has since been confirmed by many observers (20, 27, 29, 34, 38, 51, 58). Indeed, pregnancy is one physiological condition in which the blood copper content is greatly altered. In the 40th week of pregnancy the plasma copper averages about 261 ± 10.5 µg. per cent (20). The plasma copper begins to increase in the first trimester and reaches maximum values during the last trimester (Fig. 10). Normal values are then regained rapidly during the first two months post-partum.

As shown in Table 6, the increase in whole blood copper in pregnancy is due entirely to an increase in the plasma copper, with the result that the ratio of cell copper to plasma copper is greatly reduced.

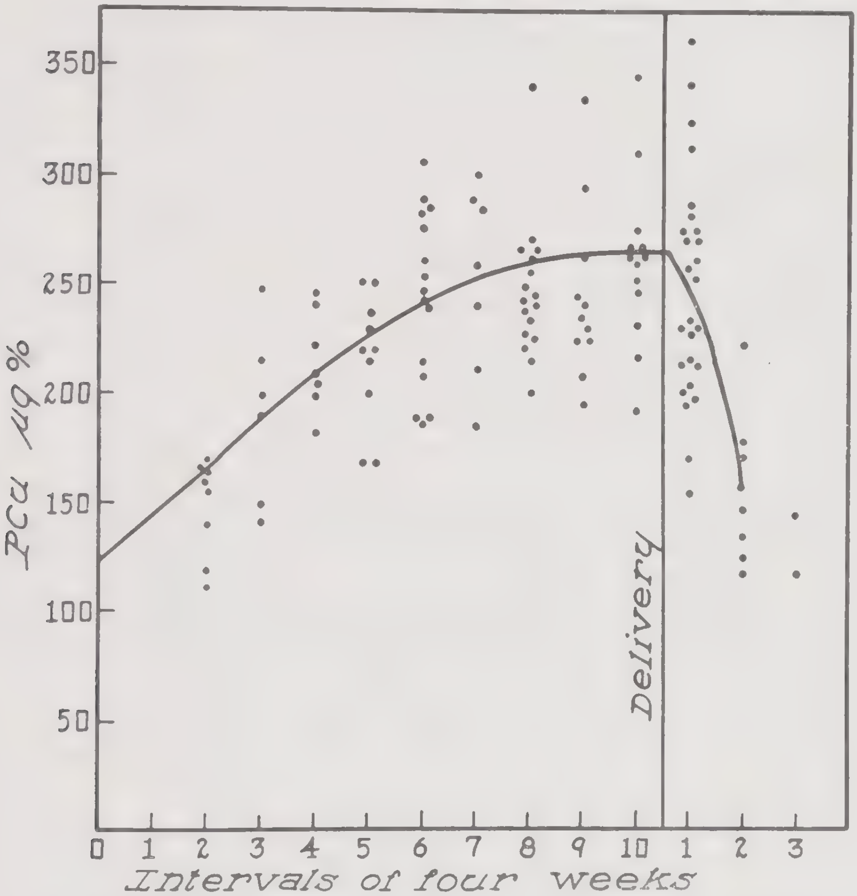

FIG. 10. Plasma copper (PCu) during pregnancy and the post-partum period. (Fay, Cartwright, and Wintrobe, *J. clin. Invest.*, 28: 487. 1949).

Thompson and Watson (68) found that in pre-eclampsia the serum copper level shows a small but significant increase over the normal elevation occurring in pregnancy. From the few cases studied, there did not appear to be a correlation between the severity of the toxemia and the height of the serum copper level.

The significance of the increased copper level in pregnancy is obscure. It has been suggested that it represents a normal, physiological adjustment for the transport of copper from the maternal blood to the fetus, but no evidence has been presented in support of this view. Indeed, although there is considerable transfer of iron from the mother to the fetus, the maternal plasma iron values are low and the fetal values high, just the reverse of the situation as regards copper (20). It seems more likely that the hypercupremia of pregnancy is associated with some more specific function. Holmberg and Laurell (32) have pointed out that the increase in serum copper in pregnancy runs parallel to the increase in the histaminolytic activity of the plasma. Thompson and Watson (68), however, found that the decrease in the histaminolytic activity of the plasma in patients with pre-eclamptic toxemia was not accompanied by any corresponding decrease in the level of serum copper.

Leukemia. In acute leukemia we have observed extremely high values for whole blood, plasma, and cell copper. The mean corpuscular copper content is markedly increased to a mean of 135 $\mu\mu\mu$g. per cell. A favorable response to the administration of adrenocorticotrophic hormone is accompanied by a decrease in the copper values, and relapse is accompanied by an increase in the copper values. This is illustrated in Fig. 11. Other observers have also found increased plasma copper values in acute leukemia (1, 27, 23).

In chronic leukemia there is a slight increase in whole blood, cell and plasma copper, but this increase is neither as great nor as consistent as in acute leukemia (Table 6).

Malignancy. Heilmeyer (27) observed an increase in whole blood and plasma copper in patients with a variety of types of malignancy. He found the erythrocyte copper to be decreased in many cases, with the result that there was a reduction in the ratio of cell copper to plasma copper. In patients with Hodgkin's disease we have observed an increase in whole blood and plasma copper and no change in the cell copper, with the result that the ratio of cell copper was decreased (Table 6).

Iron Deficiency Anemia. In patients with iron deficiency anemia there is a marked increase in cell copper and only a slight increase in whole blood and plasma copper, so that the ratio of cell copper to plasma copper is increased. The mean corpuscular copper content in this condition is increased to 80 μ μ μg. per cell. These results confirm those of Heilmeyer (27). In Fig. 12 is shown the decrease

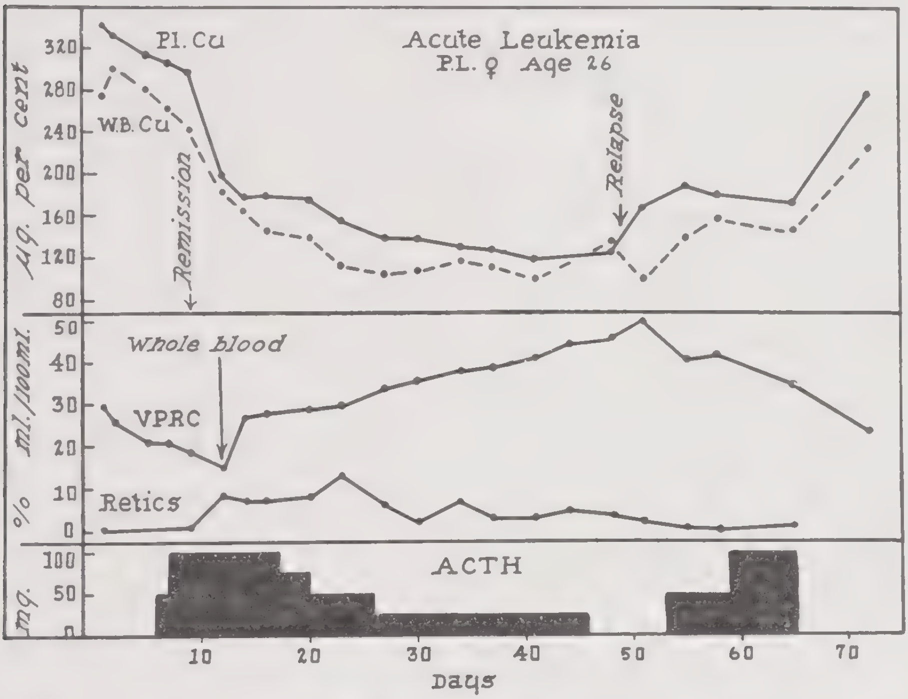

FIG. 11. Changes in plasma and whole blood copper in a patient with acute leukemia. The values were initially markedly elevated and decreased to normal as the disease responded to adrenocorticotrophic hormone (ACTH) therapy. When relapse occurred the anemia reappeared and the copper values again became elevated.

PCu plasma copper; W.B.Cu, whole blood copper; VPRC, volume of packed red cells; Retics, reticulocytes.

which took place in plasma copper in a patient with microcytic hypochromic anemia during iron therapy.

In nutritional anemia in infants and children, Axtrup (1) found normal plasma and whole blood copper values.

Pernicious Anemia. In patients with pernicious anemia we have observed a slight but proportionate increase in whole blood, plasma, and cell copper. The copper content of the macrocytes is, however, greatly increased. We have obtained values for the mean corpuscular copper content of about 100 $\mu\mu\mu g$. per cell (Table 6). This increase in copper in the cells is proportional to the increase in the cell size, so that the mean corpuscular copper concentration is unaltered.

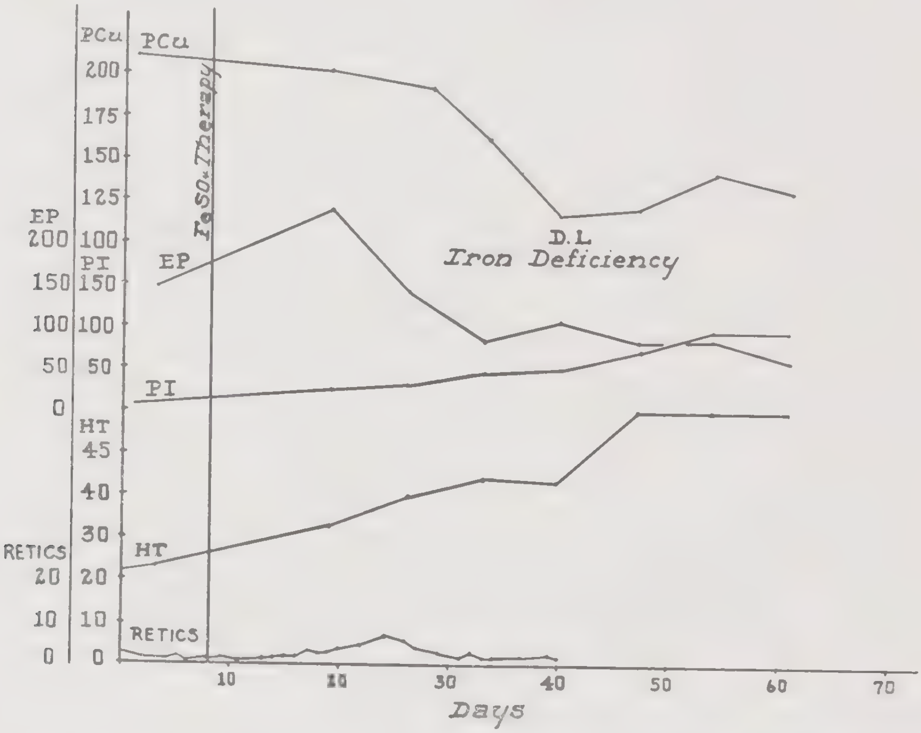

FIG. 12. Iron deficiency anemia with hypercupremia, hypoferremia, and an increase in free erythrocyte protoporphyrin. During therapy with ferrous sulfate a marked decrease in plasma copper took place. Symbols as in preceding figs. (Cartwright et al., *Blood,* 3: 501. 1948).

Miscellaneous Conditions. Increased plasma copper values have been observed in aplastic anemia, thalassemia, nephritis, various types of liver disease, and schizophrenia (27, 11). An increase in blood, urine, and liver copper has been observed in Wilson's disease (43) and Denny-Brown has observed considerable clinical improvement in some patients with this disorder following mobilization of their copper with BAL (personal communication). In spite of detailed studies, no alteration in copper metabolism has been demonstrated in multiple sclerosis (5, 43).

As mentioned previously, Heilmeyer (27), Locke, Main and Rosbash (38), and Nielsen (52) found low normal serum copper values in hypothyroidism and elevated values in hyperthyroidism, the basal metabolic rate and the copper level being somewhat correlated.

Many investigators, especially Heilmeyer (27) and Sacks (60) have noted the reciprocal relationship between blood iron and copper. It should be pointed out, however, that Sacks and his coworkers referred to total blood iron and copper. This would of course be true, since the copper content of the plasma is slightly higher than the content of the red cells. Consequently, if anemia were present there would be a decrease in *total* blood iron and an apparent increase in whole blood copper. Using the terms hyper- and hypo-ferremia and hyper- and hypo-cupremia in reference to the plasma rather than to whole blood, it is true that in many conditions, such as infections, pregnancy, and iron deficiency, the plasma iron is low and the plasma copper is elevated (11). It is not true, however, that in those conditions in which the plasma iron is high, such as pernicious anemia, thalassemia, and aplastic anemia, that the plasma copper is low (11). In the last two conditions both the plasma iron and copper are greatly elevated. Low plasma copper values have rarely been observed in any condition, as will be discussed later.

Plasma Copper in Various Experimental Anemias

Determinations of plasma copper, plasma iron, and free erythrocyte protoporphyrin have been made in our laboratory in various types of experimentally induced anemia in swine. These values are summarized in Table 7.

Pyridoxine deficiency (9) in swine is characterized by a microcytic hypochromic anemia with a slight reduction in plasma copper, a marked increase in plasma iron, and a marked decrease in free erythrocyte protoporphyrin. During the reticulocytosis following therapy there is no sharp rise and fall in plasma copper, but rather a gradual rise as the anemia is alleviated. McKibbin et al. (39) have found total blood copper at a low-normal level in anemic, pyridoxine-deficient dogs.

Chronic protein deficiency (10) in swine is characterized by a normocytic normochromic anemia, a more severe hypocupremia than in pyridoxine-deficient swine, a slight reduction in plasma iron and normal erythrocyte protoporphyrin values. The values in a single pig following therapy with protein are illustrated in Fig. 13. As the serum albumin rises there is a parallel rise in the plasma copper. The correlation between the total serum protein, albumin, and plasma copper is high, as shown in Fig. 14. The correlation between the serum globulin and plasma copper is rather poor. However, it should not be concluded from this that copper in the plasma is bound to albumin since, by the chemical separation method used, many low molecular weight globulins are separated with the albumin fraction.

TABLE 7

Plasma Copper, Plasma Iron, and Free Erythrocyte Protoporphyrin in Various Types of Experimental Anemia in Swine

Group	No. Pigs	Plasma Copper μg. %	Plasma Iron μg. %	Erythrocyte Protoporphyrin μg./100 ml. RBC
Normal	31	206 ± 4.7	169 ± 7.0	118 ± 11.5
Pyridoxine-Deficient	14	160 ± 10.4	468 ± 44.5	47 ± 3.6
Protein-Deficient	24	139 ± 5.3	115 ± 6.1	124 ± 7.3
PGA-Deficient and Protein-Deficient	3	166 ± 6.3	193 ± 11.2	70 ± 6.6
Iron Deficient	15	207 ± 6.1	31 ± 1.9	127 ± 8.1
Acute Hemorrhage	3	281 ± 8.5	24 ± 3.9	219 ± 19.1

PGA, pteroylglutamic acid.
Values are means ± standard errors.

The combined deficiency of pteroylglutamic acid and protein in swine (13) which results in the development of macrocytic anemia is characterized by plasma copper levels which are not different from those seen in protein-deficiency alone. There is a slight increase in plasma iron and a low level of free erythrocyte protoporphyrin.

Chronic iron deficiency in swine (10) is characterized by a microcytic hypochromic anemia, hypoferremia, and normal free erythrocyte protoporphyrin and plasma copper levels. Acute hemorrhage in pigs (10) is accompanied by an increase in free erythrocyte proto-

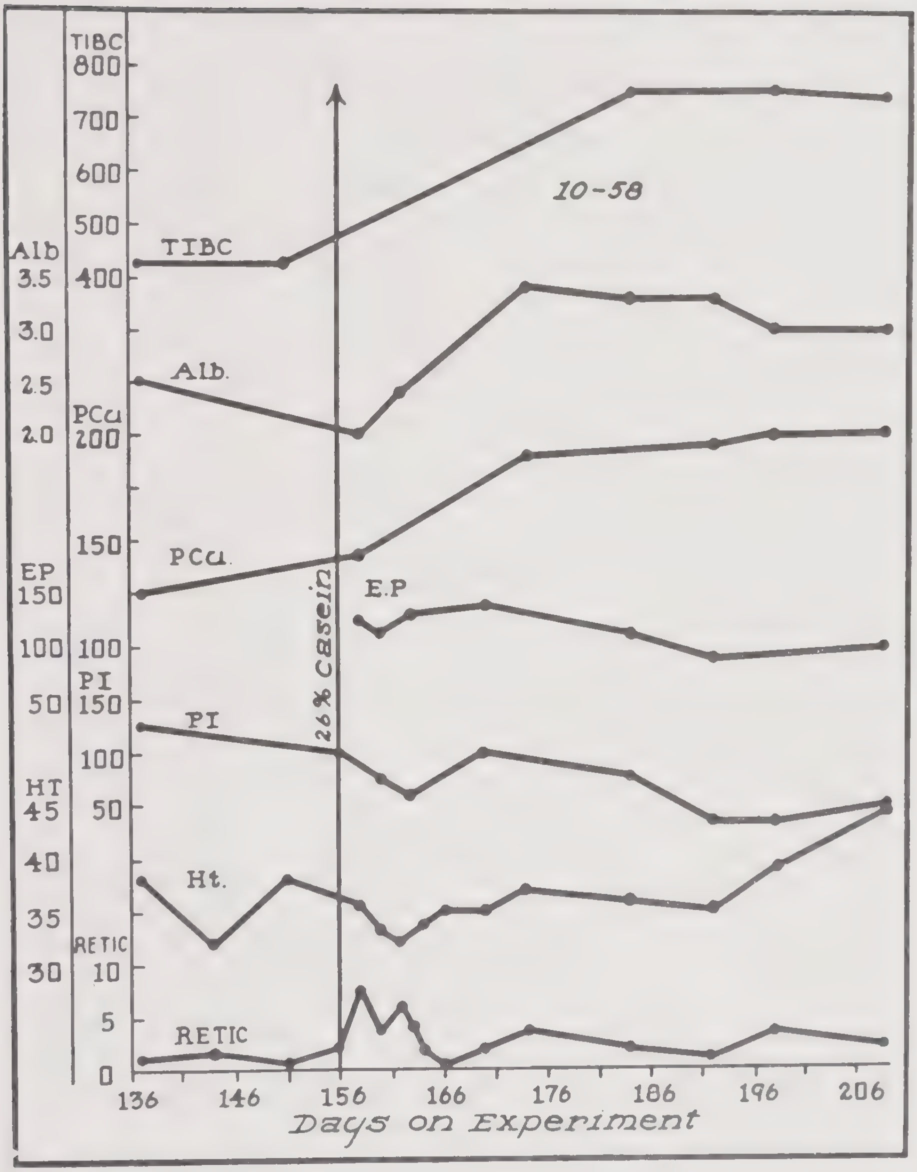

FIG. 13. The effect of protein therapy in a pig deficient in protein. As the serum albumin increased following therapy there was an increase in the plasma copper. Symbols as in preceding figs. TIBC, the total iron-binding capacity of the serum in μg. per cent. (Cartwright and Wintrobe, *J. biol. Chem.*, 176: 571. 1948).

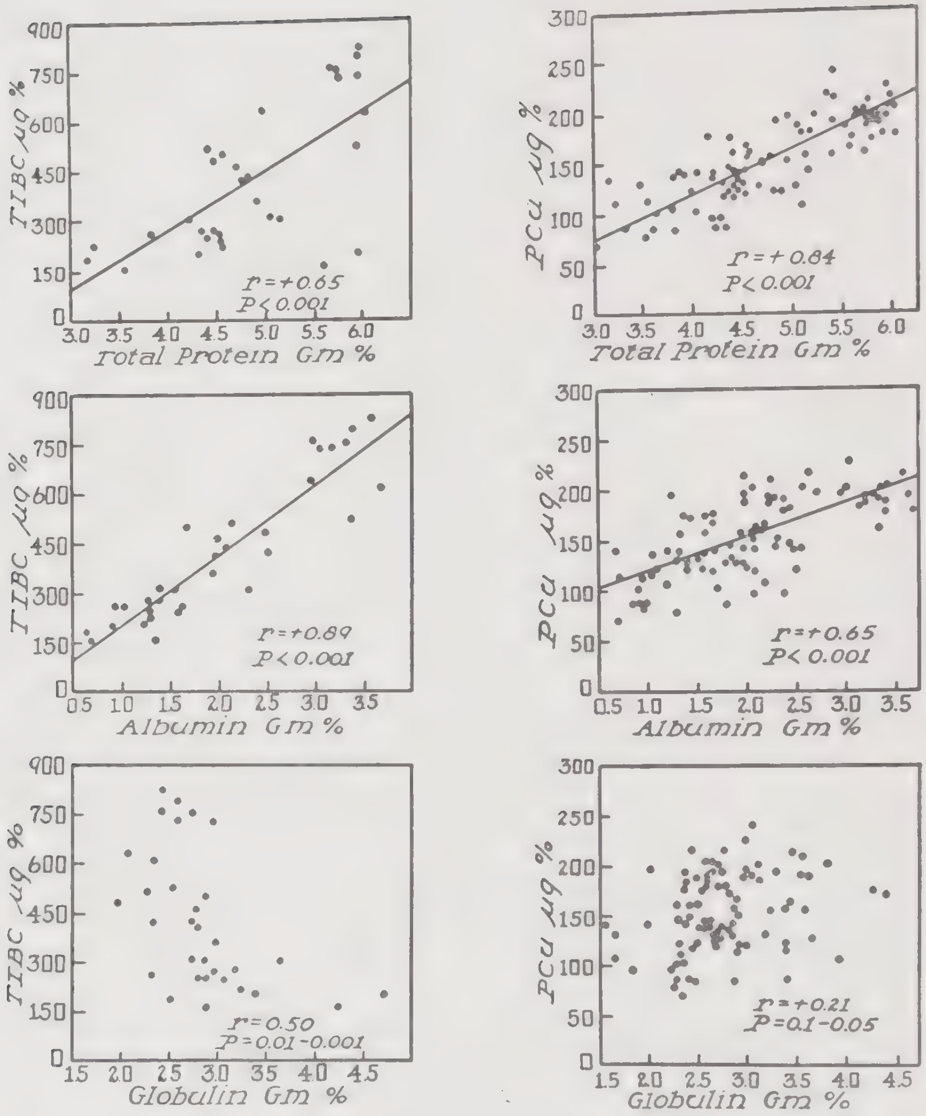

FIG. 14. The correlation between total serum protein, serum albumin, and serum globulin, respectively, with the iron-binding capacity of the serum and the plasma copper. The proteins were determined by salt fractionation and the biuret method. r, the correlation coefficient. P, the probability that a correlation as large as that indicated could occur by chance in a population in which no correlation exists. (Cartwright and Wintrobe, *J. biol. Chem.*, 176: 571. 1948).

porphyrin and plasma copper. The chemical changes in the blood of a pig from which a large quantity of blood was removed, and then a chronic deficiency of iron was induced, are illustrated in Fig. 15.

It should be pointed out that although in pyridoxine deficiency and following acute hemorrhage there is a reciprocal relationship between plasma iron and copper, in protein deficiency and in pteroylglutamic acid deficiency this reciprocal relationship does not exist.

Experimentally induced copper deficiency in pigs (66), sheep (2), and dogs (55) is accompanied by a reduction in the whole blood copper as well as in plasma copper levels. In pigs, the copper content of whole blood falls to levels as low as 7 μg. per cent and that of plasma to 4 μg. per cent. The feeding of copper results in a rapid rise. It has been suggested (66) that in pigs rapid hemopoiesis cannot take place unless the copper content of the whole blood is maintained above 20 μg. per cent. Dogs (55) made anemic by excluding iron and copper from the diet show a temporary increase in whole blood copper when iron alone is fed. The administration of copper alone is associated with a rise to normal of the blood copper level, but in time the latter falls again if the administration is not continued. If iron is then given, there is a sharp rise in blood copper, indicating that when an acceleration of hemopoiesis takes place there is a mobilization of copper from the stores to the blood. This is in accord with the observations recorded above that following hemorrhage there is an increase in blood copper. In sheep (2), anemia due to a deficiency of copper does not occur until some time after the blood level decreases to 10 to 20 μg. per cent.

Copper Deficiency in Man

No uncomplicated or clearly defined copper deficiency has ever been reported in human subjects. If it should occur, it would be expected to be accompanied by hypocupremia since, as pointed out above, animals deficient in copper have low blood copper values. Actually, hypocupremia in human subjects is extremely rare. In the many hundreds of serum or plasma copper determinations which

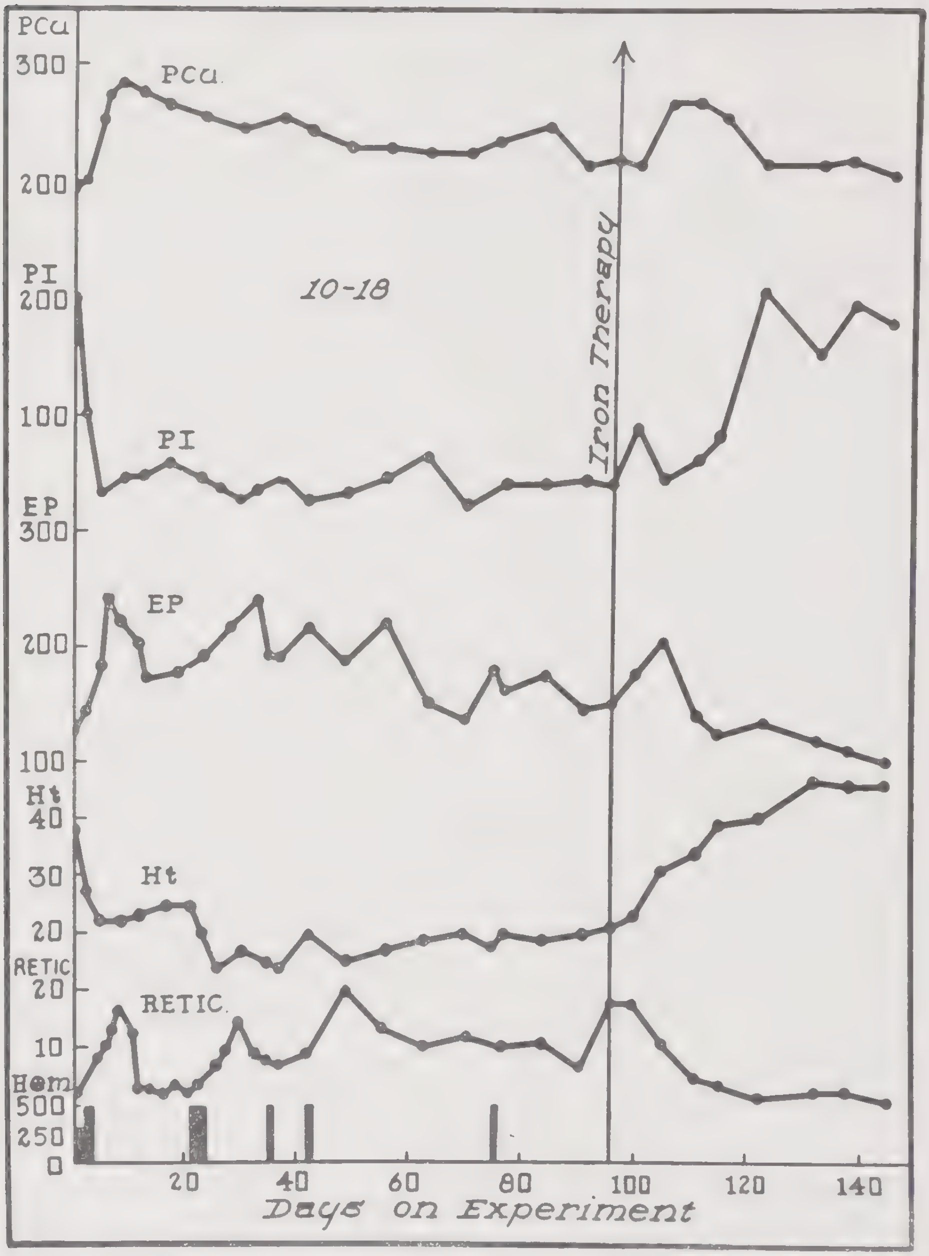

FIG. 15. The effect of phlebotomy and a diet low in iron on the blood of a pig. Under *Hem* the quantity of blood removed is expressed in ml. On the 96th day of the experiment the pig was treated with 2 g. of ferrous sulfate daily. (Cartwright and Wintrobe, *J. biol. Chem.*, 176: 571. 1948).

have been performed in our laboratory, only twice has hypocupremia been encountered. One patient had severe renal disease and marked hypoproteinemia, hypoalbuminemia, and edema. The other instance was in a patient with hemochromatosis and adequate copper in the tissues (11). Axtrup (1) has stated that he has only occasionally observed a low blood copper level in children with nutritional anemia and that copper therapy in those children has been ineffective in alleviating the hypocupremia. Cartwright and Wintrobe (6) measured the serum copper levels of 38 anemic American prisoners of World War II who had been on a grossly inadequate diet for an average of 40.1 months. In 50 per cent of these the serum copper values were within the normal range of 92 to 140 μg. per cent. In the remainder the values ranged between 140 and 215 μg. per cent. In none of the soldiers was there hypocupremia. This was in contrast to the levels of serum iron, as hypoferremia was present in 55 per cent of the group.

A combined deficiency of copper and iron has been demonstrated in an occasional child with nutritional anemia. Axtrup (1), in a study of 146 anemic infants and children, found two in whom the anemia responded poorly to adequate doses of iron and the administration of copper resulted in a reticulocytosis and an increase in hemoglobin. Oddly enough, the serum copper levels of these patients were normal prior to therapy. Müller (48) observed a reticulocytosis in one child following the administration of copper alone; and Moore (46), using the double reticulocyte method, observed a second reticulocyte peak in several anemic children following the administration of copper sulfate 2 weeks after iron therapy.

Numerous claims have been made that microcytic hypochromic anemia in human subjects responds more rapidly to combined iron and copper therapy than to iron alone. On the whole, these studies have not been well controlled. The rather voluminous and controversial literature on this subject has been reviewed recently by Axtrup (1) and by Cartwright (8). It may be summarized by saying that most investigators are in agreement that in infants and young children hemoglobin regeneration is accelerated when copper is given in addition to iron. In adults, supplemental copper therapy is unnecessary and of no demonstrable value in most patients.

It is not surprising that a deficiency of copper has not been demonstrated in adults, since the daily requirement of 2 mg. is easily supplied by even a mediocre diet, and since there is storage of copper in the tissues. In order for an adult diet to be markedly deficient in copper, the total caloric intake would have to be so low that individuals would die rather rapidly of generalized malnutrition. If copper deficiency is to be found in human subjects, it would be expected to occur in children reared solely on an unpasteurized cow's milk diet in an area of the world where the copper content of the soil is extremely low. Careful studies of anemia in children raised under these conditions have not, to my knowledge, been reported.

The Functions of Copper

The functions of copper in mammals are not known. From the results of studies in copper-deficient animals, it would seem that copper is concerned in erythropoiesis (8), in the process of myelinization of the central nervous system (2), and in the maintenance of normal mammalian pigmentation (21). In what manner copper functions in these ways is not understood.

It has been demonstrated in rats that copper has a role in the formation of cytochrome A, cytochrome oxidase, and catalase. Since these enzymes are probably essential for normal hemopoiesis, it has been suggested that copper is in this way related to erythropoiesis (67). This, however, is somewhat speculative. The proposed role of the copper protein, caeruloplasmin, in the histaminolytic system has yet to be confirmed. It has been shown that the oxidation of glutathione, cysteine, ascorbic acid, and glucose is accelerated in the presence of copper (8). The importance of these observations is unknown. Copper is an essential part of the molecule of at least three plant oxidases, namely, tyrosinase, laccase, and ascorbic acid oxidase (18). The importance of these observations for mammalian metabolism has yet to be defined.

CONCLUSIONS

Although a nutritional deficiency of copper is rarely if ever observed in man, the metabolism of copper, as indicated by changes in red cell, plasma, and whole blood copper, is greatly altered in pregnancy as well as in many pathological states. This is especially true in infectious, hemopoietic, and malignant diseases. Interpretation of the significance of these alterations is not possible because of lack of knowledge of the manner in which copper functions. It is evident, however, that copper plays an important role in the metabolism of the human body, both in health and disease. Further studies, especially in regard to the reasons for and the functions served by the metabolic changes in copper metabolism, should aid greatly in our understanding of disease and the defense of the body against it.

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Discussion

Dr. Arnon: What is the relative contribution to the human diet of copper by foods of animal origin vs. foods of plant origin?

Dr. Cartwright: That depends upon the type of animal food. Liver, of course, would be relatively high, whereas vegetables are comparatively low.

Dr. Arnon: I was very much struck by the fact of the relative status of copper and molybdenum so far as the plant is concerned. Both of these are essential for the plant, which means, of course, that no plant would grow unless it has a small amount of copper and molybdenum. Of course, the tolerance of plants to higher concentrations varies considerably. In some cases certain plants can tolerate very high concentrations of molybdenum with respect to copper, and with other plants it is just the reverse. I should think that this would greatly influence the copper and molybdenum metabolism in animals feeding on these various types of vegetation. In other words, there would seem to be no *normal* plant so far as animal nutrition is concerned.

Dr. CARTWRIGHT: I think that is a very interesting point.

Dr. VALLEE: It should be mentioned that Holmberg last summer at the International Congress of Biochemistry withdrew his statement regarding the anti-histaminolytic activity of caeruloplasmin. Have you done any work on the concentration of copper in normal compared to tumorous tissue?

Dr. CARTWRIGHT: It has been reported that in both animal and human tumors the copper content is exceptionally high, although I believe there are some investigators who do not agree with this viewpoint. I think the problem requires more work.

Dr. THOMPSON: This work you are talking about now is on the total metal content?

Dr. CARTWRIGHT: They were done both polarographically and chemically.

Dr. TALBOT: Did I understand you to say that the adrenals have no influence on copper?

Dr. CARTWRIGHT: No. What I said was that in so far as our studies have gone there is no relationship between copper content and the excretory activity of the adrenal gland.

Dr. TALBOT: Would you mind elaborating on that?

Dr. CARTWRIGHT: The administration of ACTH or cortisone to dogs does not alter the plasma copper level. In contradistinction to this, iron metabolism is greatly influenced by the adrenal.

Dr. THOMPSON: Is there any evidence that BAL will increase the excretion of copper in perfectly normal individuals?

Dr. CARTWRIGHT: Yes, there is.

Dr. VALLEE: Have you had some experience with children?

Dr. CARTWRIGHT: No, we haven't.

Dr. DAVIS: Dr. Marston, have you tried BAL with some of your sheep?

Dr. MARSTON: No. Others have and have been unable to substantiate their own claims.

Dr. CUNNINGHAM: Our experience is that large doses administered to sheep over a period of 3 weeks do not reduce liver copper.

Dr. Vallee: Have you examined any cases of multiple sclerosis with respect to copper content of the tissues?

Dr. Cartwright: Yes, we have examined the blood and found the copper content to be normal.

Dr. Arnon: I suppose you have run into cases of copper toxicity in humans?

Dr. Cartwright: Very rarely have such cases been observed.

Dr. Davis: We have run across copper toxicity in animals.

Dr. Arnon: What are the clinical manifestations?

Dr. Davis: In the cattle, copper toxicity developed, in our experience, when one gram of copper sulfate per pound of live weight per day was fed. The animals progressively cut down on their food intake and anorexia develops. When the copper level has reached about 1600 p.p.m. in the liver there is a gradual hemolysis of the blood, extreme jaundice which develops coincidentally with that, the spleen assumes a very dark coloration known as "blackberry jam" spleen, there appears to be edema in the liver, and the tissues of the liver take on a rubber-like texture. The most striking thing when the animal is opened is the yellow mustard-brown appearance of the entire animal.

Dr. Talbot: Is the spleen very much enlarged?

Dr. Davis: Apparently not.

Dr. Marston: There are similar manifestations in sheep which have succumbed to a hemolytic crisis after being subjected to conditions which result in the accumulation of a high concentration of copper in the liver.

Dr. Bear: Are you sure it is not a deficiency of some other trace element which is due to excess copper?

Dr. Davis: I suppose that Dr. Bear could argue that an excess of copper created a deficiency. However, we have no evidence in favor of that viewpoint.

Dr. Arnon: What was the diagnostic criterion in calling it copper toxicity?

Dr. Davis: Particularly the fact that we were giving the animal a high level of copper.

Dr. Arnon: Have you ever observed copper toxicity on the range?

Dr. Davis: The only place where I know of its having been observed is in Australia. Dr. Marston, would you care to describe this condition?

Dr. Marston: In experimental studies of the effects suffered by sheep which are subjected to a chronic overdosage of copper, we have observed the copper concentration in the liver is increased to ten times or more of that normally found. The animals suffer no apparent ill health until there is a catastrophic liberation of copper into the blood stream, when the blood-copper level increases sometimes to levels of more than ten times that which normally prevails. This is followed by considerable hemolysis, and the evident signs of icterus appear. English breeds of sheep usually succumb to a crisis of this sort as their kidneys become blocked with hemoglobin. Under identical conditions Merino sheep are better able to excrete the hemoglobin and so survive. Considerable liver damage results, and after suffering two or three crises, the Merino usually succumbs as a consequence of impaired liver function.

Dr. TALBOT: Will fasting bring on the crisis?

Dr. MARSTON: Yes. Fasting can bring on a crisis, but other factors also can be responsible.

Dr. ARNON: This condition which you have just described was experimentally induced, was it not?

Dr. MARSTON: Yes, that is true.

Dr. ARNON: Have you ever run into copper toxicity in sheep on the range in Australia?

Dr. MARSTON: Yes. Sheep grazed on certain natural pastures in Australia, or at least on pastures which have not been subjected to manurial dressings of copper, tend to accumulate copper within their livers and suffer a fate identical with that which results when an abnormal copper storage has been induced by experimental means. The fodder available to the flocks pastured on these areas contains no greater concentration of copper than that which grows on other areas where the malady is never experienced. There is a tendency to indict the relatively low molybdenum content of the affected pastures, the hypothesis evidently being based on the contention that the copper stored in the liver is regulated by the molybdenum content of the fodder. The evidence for this, however, is equivocal. Recently liver damage supervening on the chronic ingestion of a species of heliotrope (*Heliotropium europaeum*) which grows on the affected areas entered by the grazing stock, has been shown to be, at least in part, responsible for many of the deaths previously attributed to enzootic toxic jaundice. There is no evidence of which I am aware that suggests that fodder plants can accumulate sufficient copper in their tissues to induce *per se* the effects of copper poisoning in the animal. Other factors are certainly involved in the etiology of enzootic toxic jaundice, but this question is by no means finally answered.

Dr. ARNON: All of the conditions which Dr. Davis described in Florida are experimentally induced?

Dr. Marston: Yes, I think that is true. Actually, the toxic jaundice syndrome was first described in France, when it appeared in sheep that had grazed in an orchard which had been sprayed with Bordeaux mixture.

Dr. Cunningham: It may be of interest to mention the possible influence of copper in causing the black kidneys that are a characteristic feature of copper poisoning. In the terminal phase of copper poisoning, blood copper is elevated to about ten times normal, i. e., to about 1 part in 100,000. If normal kidney is treated, in vitro, with a solution of copper of this strength in normal saline, blackening is produced. It seems possible that the copper stimulates some enzymatic process which causes the pigment formation.

THE COPPER AMALGAM ELECTRODE AND ITS APPLICATIONS

IV. THE CHEMICAL NATURE OF THE COPPER COMPLEXES IN PEAT SOILS AND PLANTS

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The occurrence of plants exhibiting symptoms of copper deficiency on soils that contain copper and are exposed to contamination with copper is evidence that soils can react with this element to form either insoluble compounds or complexes. This fixation of copper has been studied by measuring the electromotive forces of cells of the following type:

$$\mathrm{Hg}\,;\,\mathrm{Hg_2Cl_2\,(S)},\,\mathrm{KCl\ (Sat.)}:\,:\mathrm{Cu^{++}\ (Peat\ Suspension)}\,;\,\mathrm{(Hg)\,Cu}$$

The copper electrode reaction is $Cu^{++} \rightarrow Cu$ (metal). Thus, the electromotive force of the cell, neglecting the liquid junction potential, is given by the following equation:

$$E = E_0 - \frac{0.059}{2} \log_{10} \frac{1}{(Cu^{++})}\,.$$

E is the observed potential, and E_0 is the standard potential of the cell, 0.099 volt. (Cu^{++}) is the molar activity of the cupric ion. For convenience, these cupric ion activities are expressed as pCu numbers which are defined as $-\log_{10}$ of cupric ion activity.

The reliability of the electrode was checked in three ways (2). First, the solubility products of two insoluble copper compounds were measured and found to compare satisfactorily with known values. Second, the salts contained in water extracts of peat soils were diluted with water while maintaining cupric ion molality constant. The ionic strengths of these solutions calculated from the Debye-Huckel law were consistent with the dilutions. Third, the quantities of several ions required to change the potential of a typical peat soil suspension were determined. These quantities indicated that mercuric ions, although the most probable source of error, do not affect the readings taken with the amalgam used in this investigation when added in amounts less than 0.5 m.e. per 100 g. soil. Other amalgams have been prepared which are much more sensitive to this trouble. The sensitivity to mercuric ions was expected from a consideration of standard electrode potential values.

TABLE 1

Cupric Ion Activities (pCu Values) of Primary and Secondary Copper Complexes Occurring in Peat Soils and Plants

Complexes occurring in	Cupric ion activities (pCu values)	
	Primary complexes	Secondary complexes
Peat soils	5.6 and 6.5	8.9, 11.7, 14.2, 17.1
Plant materials	5.6 and 6.5	8.9, 11.7, 14.2, 17.1

The reason for the variation in sensitivity to mercuric ions cannot be explained satisfactorily. Although these tests indicate that the potentials of the cells measured in these studies can be attributed to the reduction of cupric ions to copper metal, the low resistances of the cells indicate that other reactions may have been conducting some of the current through the cells. If other reactions were responsible for the conduction of a part of the current flowing through these cells, these reactions were also contributing to the potentials of the cells. A procedure was not found whereby these two portions of the observed potentials could be separated.

When suspensions of peat soil and plant material were titrated with copper sulfate, regions of the titration curves buffered with respect to cupric ion activity were observed. This buffer action has been attributed to the formation of complexes (3, 10). The complexes formed by titration with copper sulfate have been named primary complexes. The pCu values for the primary complexes observed in peat soils and plant materials are presented in Table 1. Typical titration curves showing the nature of this buffer action are

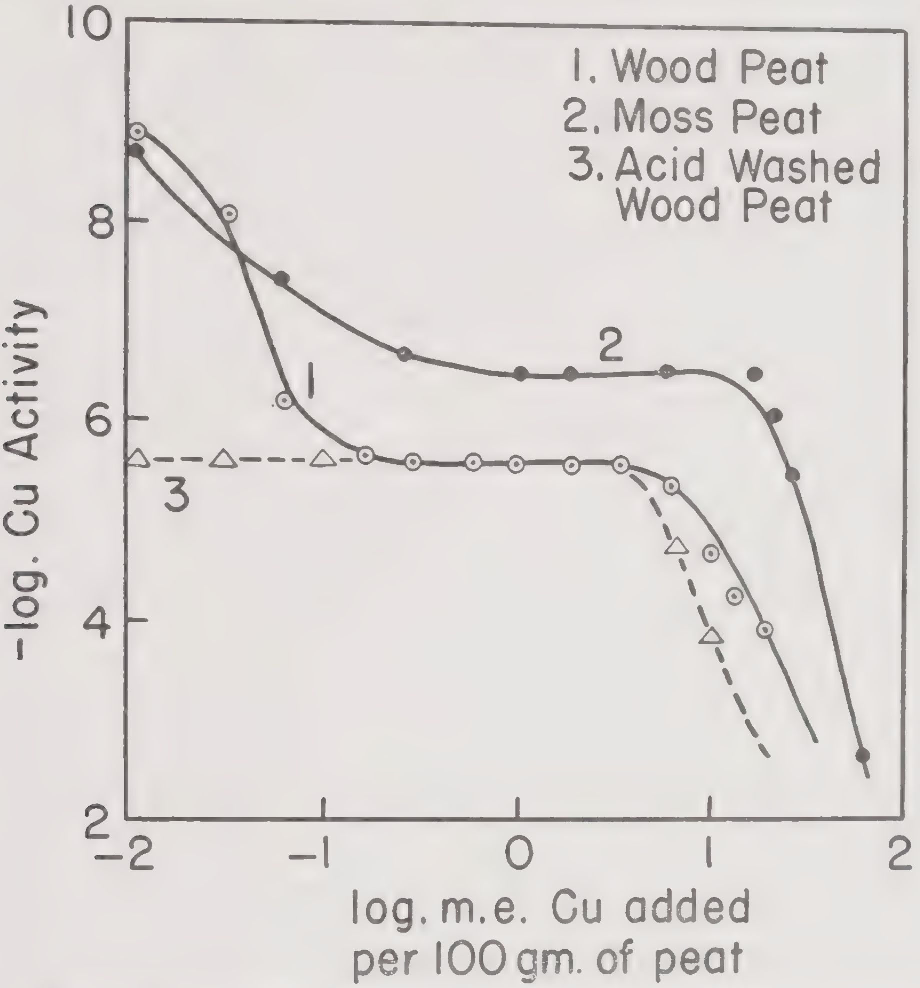

FIG. 1. Primary complexes formed by titration of suspensions of peat soil with copper sulfate.

presented in Fig. 1. When any peat soil or plant material is leached with dilute hydrochloric acid, the cupric ion activity of a suspension of the soil or material changes to that of the primary complex formed by titration with copper sulfate. In natural peat soils and plant material, this change is an increase in cupric ion activity even though small amounts of copper are removed during the leaching process. This indicates that the acid-leaching process removes something from peat soils and plant materials that is capable of reducing cupric ion activity below that of the primary complexes. This material has been identified as ammonia. When the primary complexes of peat soils and many plant materials are titrated with ammonia, four regions of the curves are buffered with respect to cupric ion activity. This buffer action has also been attributed to the formation of complexes (3, 10). These complexes have been called secondary, since they are formed by reacting the primary complexes with ammonia. The pCu values of the secondary complexes observed in peat soils and plant materials are presented in Table 1. Although, all of the secondary complexes included in Table 1 have been observed in all peat soils (10), some non-leguminous plant materials, particularly tissues deficient in nitrogen, have contained only two or three of them.

The object of this study was to determine the chemical nature of these primary and secondary complexes of peat soils and plant materials and to synthesize complexes having their cupric ion activity properties.

The work of Waksman et al. (11, 12, 13, 14, 15) and of others has shown that soil organic matter is mainly a residue derived from lignin and protein. Gottlieb and Hendricks (4) in a recent study concluded that condensation of lignin occurs in the soil, with production of fused ring structures. From methylation studies they were able to prove the presence of phenolic OH groups in soil organic matter. Macheboeuf and Viscontini (7, 8, 9) have reported that copper can form complexes with proteins in acid or alkaline solutions. They attribute the formation of a copper complex to definite reactive groups of the proteins. Since phenolic OH groups. protein residues, and various fused ring structures have been found in soil organic matter, preliminary investigations were carried out by reacting copper with lignin and with phenols and protein under different conditions. The studies were then extended to materials of known chemical composition. From several combinations of these materials, copper complexes of the same pCu values as those occurring in soils and plants were synthesized in the laboratory. It was possible from these studies to identify the copper-fixing groups in peat soils and in plants, and to explain many of the peculiar properties of copper in these systems.

REACTION OF COPPER AND AMMONIA WITH ALBUMIN AND PHENOLS

The copper activity of a one per cent solution of egg albumin was measured as a function of added copper. The results are presented graphically in Fig. 2. The albumin formed a copper complex with an activity of pCu 5.9, which is near the activity of the primary complex formed by copper and wood peat. About eight mg. of copper were fixed at this activity level by 0.5 g. of albumin.

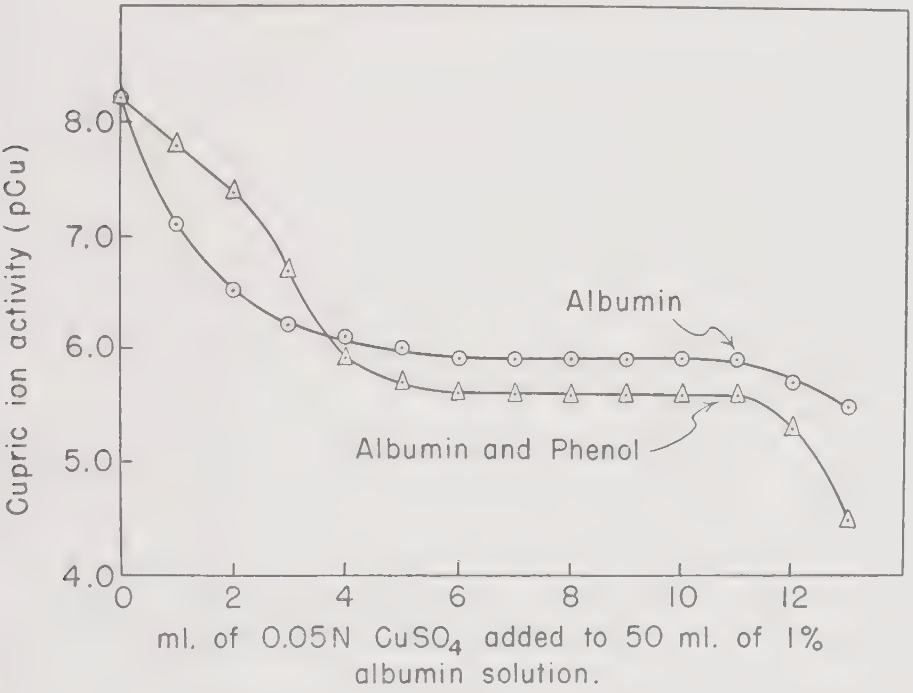

FIG. 2. Cupric ion activity of albumin solution titrated with $CuSO_4$ in absence and presence of phenol.

The activity of the complex formed was the same whether the albumin was initially at a pH of 4 or 7. But the amount of copper required to bring the protein to this activity level at pH 7 was almost double that required at pH 4. The addition of phenol to the albumin solution, prior to copper, had a marked effect on the activity of the protein-copper complex. The cupric ion activity of the complex formed in this case was increased to pCu 5.6, the value observed for wood peats. The amount of phenol required to produce this effect was extremely small. One-third of the pCu 5.9 complex was converted to pCu 5.6 by 0.1 millimol of phenol; 0.3 more millimols of phenol completed the conversion, and additional phenol had no effect. The displacement of the activity curve in the presence of added phenol is shown in Fig. 2. It is indicative of a definite reaction between protein and phenol. Several other compounds containing phenolic OH groups were added to albumin and observed to substitute for the phenol with identical results. These data are given in Table 2.

TABLE 2

Effect of Phenolic OH Group on the pCu Value of Albumin Copper Complex

Compound added to albumin solution*	pCu value of copper albumin complex†	
	In absence of phenol	In presence of phenol
Phenol	5.9	5.6
Resorcinol	5.9	5.6
Pyrogallol	5.9	5.6
Phloroglucinol	5.9	5.6
Salicylic acid	5.9	5.6
Hydroquinone	5.9	5.6
1-2-5-8-tetrahydroxy anthraquinone	5.9	5.6

* In amounts varying from 0.5 to 5 millimols per 0.5 g. of albumin.
† 1 per cent water solution of albumin.

Even though albumin in the presence of phenol gave a primary copper complex of pCu 5.6, this complex did not show the characteristic ammonia reactions of the corresponding primary peat-copper complex. This indicates that the albumin-phenol-copper complex lacked certain groups with which ammonia reacts in the peat-copper complex.

Next a lignin residue was extracted from a peat soil by refluxing it with four per cent alkali for five hours and precipitating the filtered alkali extract with hydrochloric acid. This alkali-lignin was filtered off and oven-dried. Alone, it had no copper-complexing capacity at the pCu values in question. The material was dissolved in a small amount of alkali and added to a solution of albumin. After an adjustment of pH this solution had the same copper activity properties as the phenol albumin solution. Then the mixture was boiled for five hours in two per cent HCl until most of the protein was hydrolyzed. The hydrolyzed mixture was reprecipitated by adjusting its pH. The precipitate was dissolved in alkali and

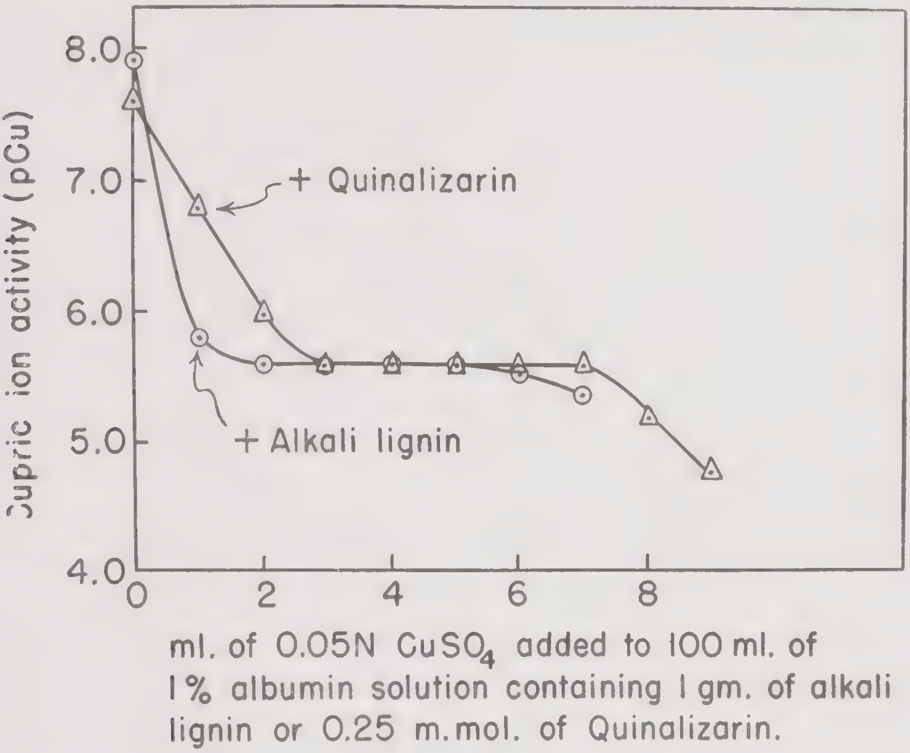

FIG. 3. Cupric ion activities of albumin partially hydrolyzed in presence of either alkali lignin or quinalizarin and titrated with $CuSO_4$.

added to a fresh amount of protein solution. The pH of the mixture was adjusted and the hydrolyzed alkali-lignin-protein material separated. A suspension of this material in water gave, upon titration, both the copper complex of pCu 5.6 as well as all four

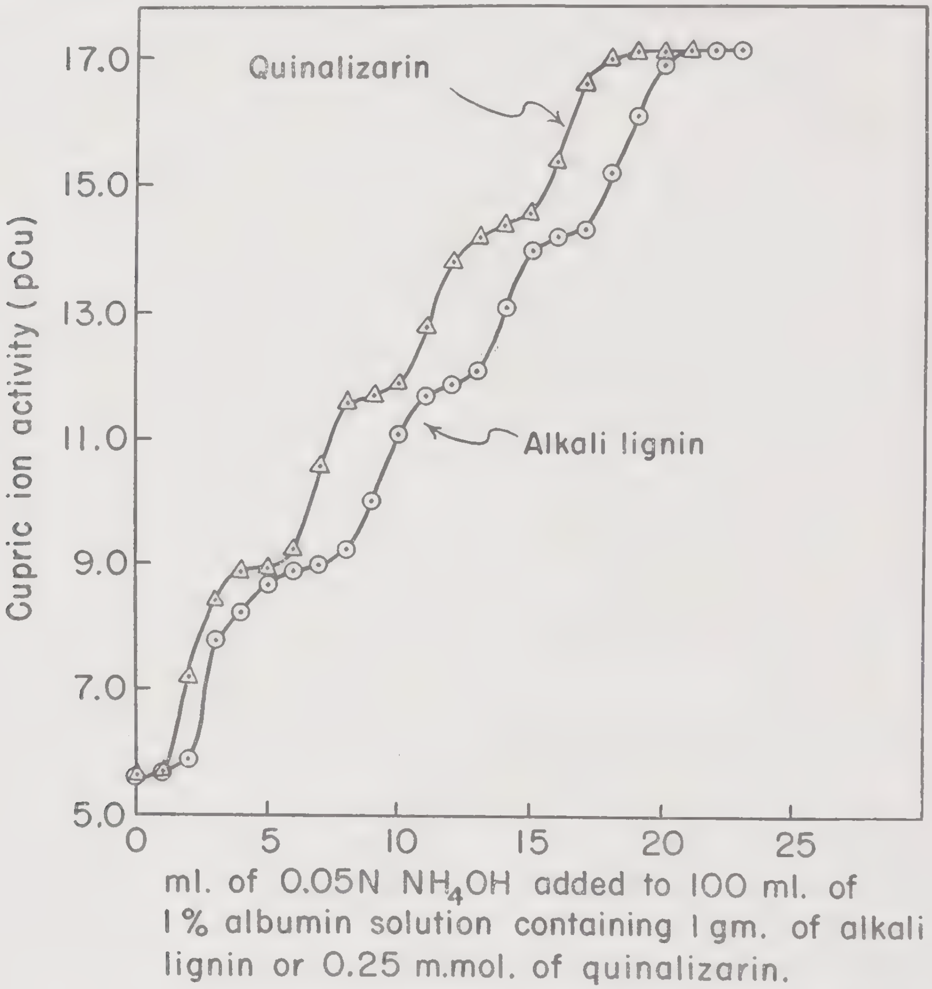

FIG. 4. Cupric ion activities of albumin partially hydrolyzed in the presence of either alkali lignin or quinalizarin and titrated with NH_4OH.

secondary ammonia complexes. Results are presented graphically in Figs. 3 and 4. The curves are identical with those of peat soils and plants (3, 10). Thus, the copper-fixing groups of peat are contained in the lignin- and protein-derived fractions of the organic matter.

The alkali-lignin was next replaced by a compound of known chemical structure. Quinalizarin, 1-2-5-8-tetrahydroxy anthraquinone, was first used instead of the alkali-lignin. This compound was selected because of its fused ring structure, solubility in alkali, and insolubility in dilute acids. It was boiled with protein in a two per cent HCl solution and reprecipitated with base. The precipitate was dissolved in a minimum amount of alkali, added to fresh albumin solution, and reprecipitated at pH four. The precipitate that came down closely resembled soil organic matter, in that it formed both the copper and the copper-ammonia complexes. These data are also presented in Figs. 3 and 4.

Thus the protein- and lignin-derived components of soil organic matter are responsible for both copper and copper-ammonia complex formation in peat soils. Also, phenolic OH groups can react with albumin and form a material that will fix copper at the same activity as the primary wood peat complex. However, this copper complex will not react with ammonia in the manner of a peat soil. If a part of the albumin is hydrolyzed in two per cent HCl, this property is developed. Digestion of albumin in HCl probably converts many peptide linkages into aliphatic primary amine and carboxyl groups. One of these groups may then be involved in the ammonia reactions.

The data presented in Fig. 2 also indicate that some group occurs in protein that will react with copper to form a complex whose cupric ion activity will change in the presence of phenol. Since cupric copper is particularly susceptible to donor-acceptor bond formation, it may be that the phenol reacts with a protein group, enabling it to form such a bond with the copper. For example, could phenol react with a peptide linkage to form a tertiary aromatic amine which might form a covalent bond with copper? These possibilities were investigated by titrating various aromatic amines and amino acids first with copper sulfate and then with ammonium hydroxide.

REACTION OF COPPER AND AMMONIA WITH AMINO ACIDS AND AROMATIC AMINES

Cysteine

Cysteine forms a cuprous mercaptide with copper. The copper activity of this cuprous mercaptide cannot be measured by means of the amalgam electrode, since the readings are unstable because of the non-equilibrium ratio of cupric and cuprous copper. However, the addition of a small amount of primary, secondary, or tertiary aromatic amine to the cysteine solution stabilizes the potential readings so that copper titrations can be made. The addition of the amine, besides stabilizing the potential, gives a copper complex when titrated with copper sulfate. Diphenylamine, when dissolved in alcohol and added to cysteine, produces a copper complex having a cupric ion activity equivalent to pCu 6.5. Thus, this complex has the same pCu value as the complex observed in moss peat (10).

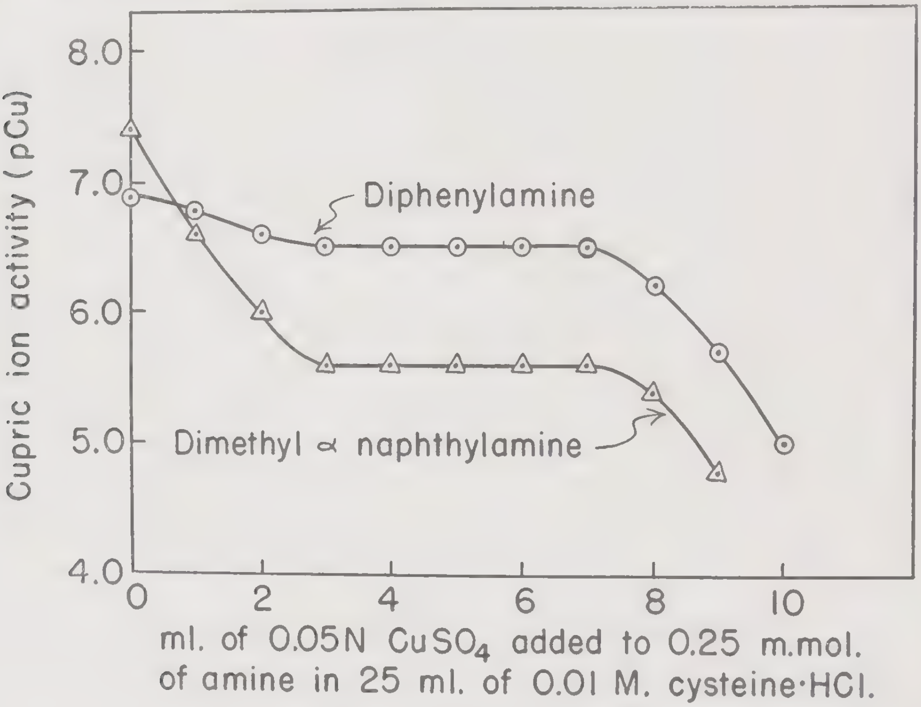

FIG. 5. Cupric ion activities of cysteine mixed with either secondary or tertiary aromatic amines and titrated with $CuSO_4$.

The diphenylamine-cysteine-copper complex also gives all of the characteristic ammonia reactions. These results are presented graphically in Figs. 5 and 6. The substitution of dimethyl-α-naphthylamine, a tertiary aromatic amine, for diphenylamine gave different results. The cupric ion activity of the primary copper complex formed, in this case, was pCu 5.6, the value observed for wood peats (10). This complex also gave the characteristic ammonia titration curve. These data are also presented in Figs. 5 and 6. α-Naphthylamine, while stabilizing the potential readings, gave neither a primary

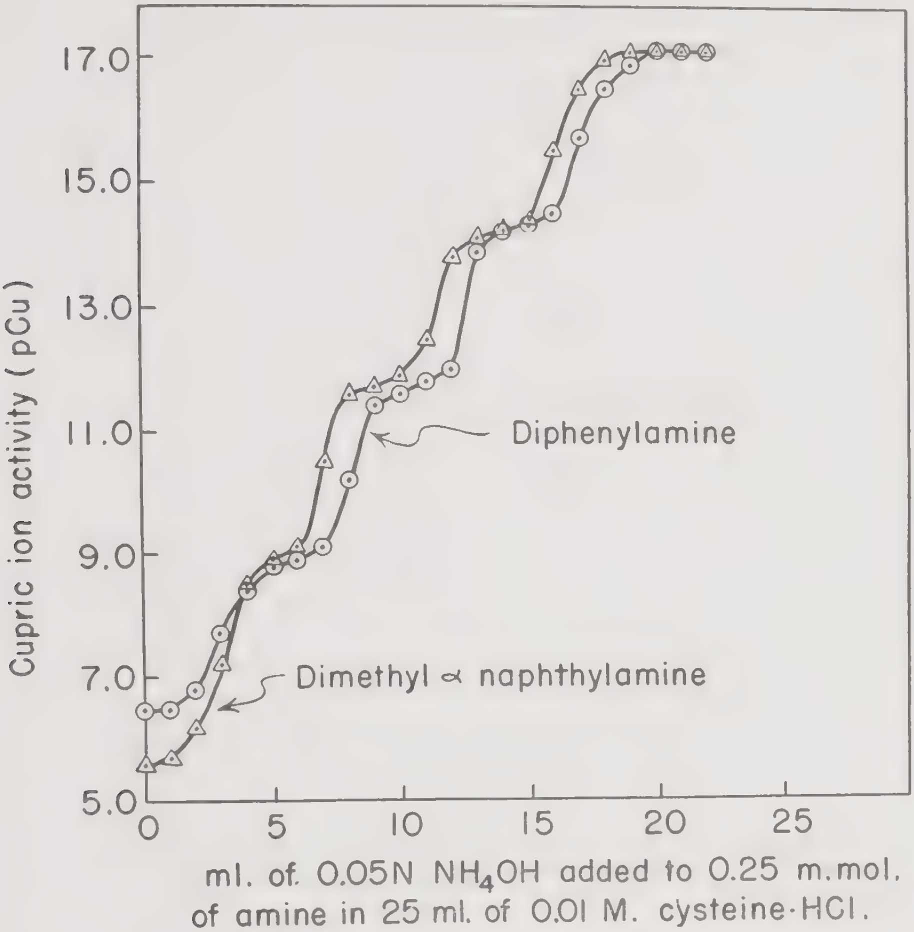

FIG. 6. Cupric ion activities of cysteine mixed with either secondary or tertiary aromatic amines and titrated with NH_4OH.

copper complex nor the reactions with ammonia. None of the primary aromatic amines tried formed either of the primary copper complexes or any of the secondary ammonia complexes.

Secondary and tertiary aromatic amines were prepared from 1-2-5-8 tetrahydroxy anthraquinone by reacting this material, dissolved in sulfuric acid, with methylamine hydrochloride and with dimethylamine hydrochloride, respectively. Solid amines were obtained upon dilution with water. These amines, when suspended in a solution of cysteine, gave the characteristic copper and ammonia titration curves presented in Figs. 7 and 8. These indicate that the property of forming primary copper complexes of the cupric ion activities in question is not limited to any particular aromatic amine but rather to two classes of these amines.

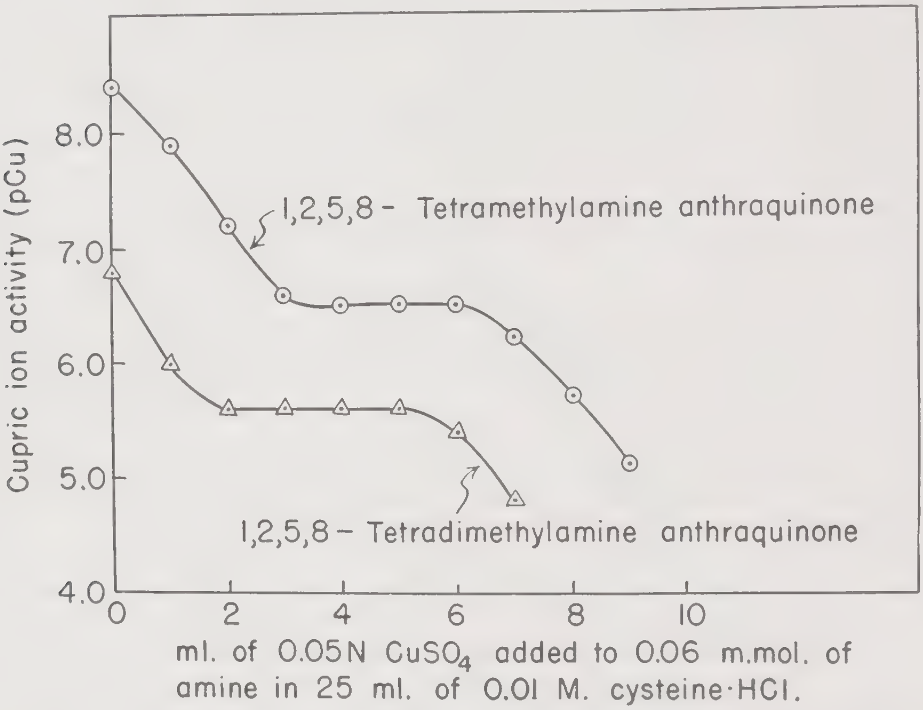

FIG. 7. Cupric ion activities of cysteine mixed with either secondary or tertiary aromatic amines, prepared from tetrahydroxy anthraquinone and either methyl or dimethyl amine, upon titration with $CuSO_4$.

Activity studies were then conducted on the complexes formed by various aromatic secondary and tertiary amines and a few other nitrogenous compounds in combination with cysteine. The data obtained on these materials are summarized in Table 3. The quantity of amine used was one amine group per molecule of cysteine. The amines were usually added to the cysteine as hydrochlorides.

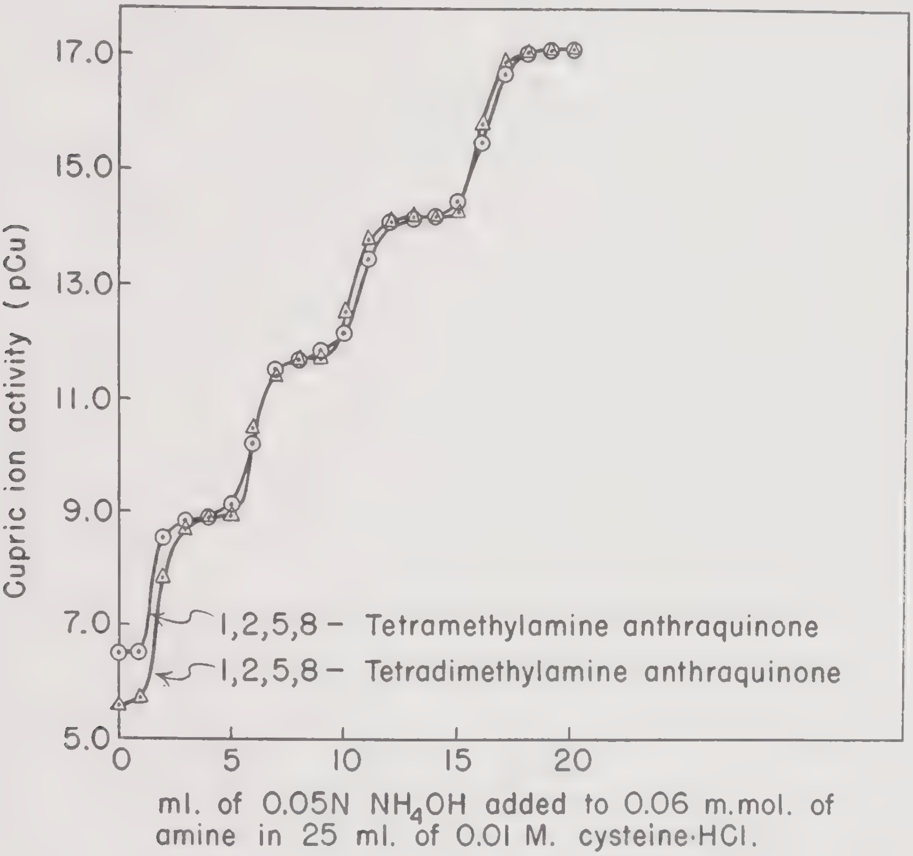

FIG. 8. Cupric ion activities of cysteine mixed with either secondary or tertiary aromatic amines, prepared from tetrahydroxy anthraquinone and either methyl or dimethylamine upon titration with NH_4OH.

The data presented in Table 3 indicate that the property of forming primary copper complexes of pCu 5.6 or 6.5 is dependent upon the type of *aromatic* amine. The latter restriction is made because the aliphatic amines did not form copper complexes. Secondary aromatic amines gave complexes of pCu 6.5, whereas tertiary aromatic amines formed complexes of pCu 5.6. All of the ethyl-amines failed to form secondary ammonia complexes, in contrast to the methylamines and the phenylamines, all of which formed four secondary complexes. Pyridine and 8-hydroxyquinoline also failed to form secondary copper-ammonia complexes. Histidine and proline failed to form either secondary or primary copper complexes of the types found in peat soils and plants. It may be worth noting that these two amines were the only ones studied that contained carboxyl groups. Thus, they may have existed largely as zwitterions rather than as amines capable of donor-acceptor bond formation with cupric ions.

TABLE 3

Cupric Ion Activities of Copper Complexes Formed from Secondary and Tertiary Amines in Combination with Cysteine

Amine	Cupric ion activity (pCu value)	
	Primary copper complex	Secondary copper ammonia complexes
1-2-5-8-tetramethylamine anthraquinone	6.5	8.9, 11.7, 14.2, 17.1
Diphenylamine	6.5	8.9, 11.7, 14.2, 17.1
Ethyl-α-napthylamine	6.5	none
Ethyl aniline	6.5	none
1-2-5-8, tetradimethylamine anthraquinone	5.6	8.9, 11.7, 14.2, 17.1
Dimethyl-α-naphthylamine	5.6	8.9, 11.7, 14.2, 17.1
Dimethylamine	none	none
Methylamine	none	none
Pyridine	5.6	none
8-hydroxyquinoline	5.6	none
Histidine	none	none
Proline	none	none

Alanine

The amino acid alanine forms more than one complex with copper. However, no complex of pCu 5.6 or 6.5 was formed in the presence or in the absence of aromatic amines.

Cystine

Cystine forms more than one copper complex, but none with an activity corresponding to pCu 5.6 or 6.5 in the presence or absence of aromatic amines.

REACTIONS OF COPPER AND AMMONIA WITH THIOGLYCOLIC ACID AND ETHYL MERCAPTAN

In order to differentiate between the various reactive groups responsible for the formation of complexes with fixed copper activity, experiments were carried out using thioglycolic acid and ethyl mercaptan. The thioglycolic acid in the presence of any aromatic secondary or tertiary amine produced copper complexes of pCu 6.5 or 5.6, respectively. However, on titration with ammonia these copper complexes failed to form all the usual secondary, copper-ammonia complexes. There was evidence for only two copper-ammonia complexes. These complexes had copper activities equivalent to pCu 8.9 and 11.7. As thioglycolic acid lacks only the $\mathrm{H\overset{|}{\underset{|}{C}}NH_2}$ group in comparison with cysteine, it seems possible that the -NH$_2$ groups are responsible for the two ammonia complexes of pCu 14.2 and 17.1. This was confirmed by the fact that addition of alanine to the system and continued titration with ammonia caused the formation of these two complexes. Thus thioglycolic acid in the presence of alanine behaved exactly as cysteine.

Ethyl mercaptan, on the other hand, formed only the cuprous mercaptide when copper was added. No complex formation occurred in this case in the presence of aromatic amines. Introduction of the carboxyl group into the system, through addition of propionic acid, did not alter the properties of the system as far as formation of any copper complex was concerned.

These data indicate that the basic groups reacting with copper in these complexes are RSH and RCOOH and that both groups probably must occur in the same molecule. These complexes also contain an aromatic amine group. This amine is of the secondary type in the pCu 6.5 complex and of the tertiary type in the pCu 5.6 complex. The R-NH$_2$ group is essential for formation of two of the secondary complexes, those at pCu 14.2 and pCu 17.1. Also, the secondary ammonia complexes may not involve the aromatic amines, since their cupric ion activity values are independent of the type of amine occurring in the primary complex. The latter point was confirmed for the pCu 17.1 complex by titrating a cysteine hydrochloride solution to pH 8.3 with ammonia, adding copper sulfate, and adjusting the pH back to 8.3 with ammonia. This solution had a pCu value of 17.1, which was stable.

Seven of these complexes were next synthesized and purified for chemical analysis.

Preparation and Composition of Primary Copper Complexes

When copper sulfate was added to a solution of cysteine and either secondary or tertiary aromatic amine, a precipitate formed and the cupric ion activity of the solution increased. The precipitate had a copper content of 44 per cent, equal to that of the cupric salt of cuprous mercaptide. It continued to form, and cupric ion activity continued to increase as copper sulfate was added, until the pCu value became constant at 6.5 or 5.6. Further additions of copper formed more precipitate, but its copper content decreased as long as the pCu value remained constant at either 6.5 and 5.6. This indicates that the precipitate was probably a mixture of the desired complex and the cupric salt of cuprous mercaptide. Separation of the pure complex from the mixture was not accomplished. Instead, a new method of synthesis was attempted.

An ammoniacal solution of cysteine was added to the cysteine-aromatic amine solution, and the pH of the solution was adjusted to a value of 8.3. The ratio of cysteine to cystine in this solution was 6: 1. It was then titrated with copper sulfate. First, a soluble violet-colored complex having a pCu value of 16.0 was formed. The amount of this complex formed could be increased by increasing the amount of cystine added. The addition of more copper sulfate lowered the pCu value through a second complex region at either pCu 6.5 or 5.6. A precipitate formed in the latter regions. This solid phase was removed, washed with alcohol to remove excess amine, washed with sodium acetate-acetic acid, buffered at pH 4.8, to remove ammonium ions, washed with distilled water and oven-dried at 70°C. When this material was suspended in water it had a pCu value of 6.5, if a secondary aromatic amine were used, or of 5.6, if a tertiary aromatic amine were used. If the aromatic amines were methyl or phenyl amines, all four secondary complexes were observed upon titration with ammonia, as indicated in Table 3.

Seven copper complexes were prepared by the preceding method, using seven different aromatic amines. These complexes were analyzed for Cu, S, and N. These data are given in Table 4.

TABLE 4

Chemical Composition of Cysteine-Amine-Copper Complexes

Kind of amine	Cupric ion activity	Per cent sulfur		Per cent nitrogen		Per cent copper	
		Observed	Calculated	Observed	Calculated	Observed	Calculated
Ethyl-α-napthylamine (Formula weight of complex, 600)	6.5	10.7	10.7	7.06	7.00	31.2	31.8
Diphenylmanine (598)	6.5	10.8	10.7	7.03	7.03	32.2	32.0
Ethylaniline (550)	6.5	11.4	11.6	7.65	7.64	35.6	34.8
Diethylaniline (578)	5.6	11.0	11.0	7.34	7.27	33.7	33.1
Diethyl-α-napthylamine (628)	5.6	10.3	10.2	6.78	6.69	30.0	30.4
Dimethyl-α-napthylamine (600)	5.6	10.8	10.7	7.03	7.00	31.9	31.8
Pyridine (508)	5.6	12.3	12.6	8.33	8.27	37.6	37.3
Percentage of variability accounted for by calculated values		99		99		96	
F observed		0.5		13.3		0.1	
F at odds of 19:1		5.99		5.99		5.99	

Inspection of the data shows that the ratio of Cu: S: N in these complexes is 3: 2: 3. Thus the ratio of copper atoms to cysteine molecules to aromatic amine groups is 3: 2: 1. This point was confirmed by dissolving the complex prepared from dimethyl-α-napthylamine in hydrochloric acid and determining the amine directly by use of a Beckman ultraviolet spectrophotometer. Formula weights and percentages of Cu, S, and N were calculated for these complexes, using the above reacting proportions. These values are also given in Table 4. They agree with the observed values within the analytical errors inherent in the methods used. It must, however, be admitted that there are statistically significant but very small differences between the observed and calculated N values. These may have been due to incomplete removal of ammonium ions from these complexes.

SUMMARY AND CONCLUSIONS

The groups occurring in peat soil and plants that might react with copper to form the two primary copper and four secondary copper-ammonia complexes previously described were studied using an amalgam electrode for measuring cupric ion activity. First, the reactions of copper and ammonia with albumin and either phenols or alkali-extracted lignin were studied. The data that were obtained showed that some combination of protein and lignin, involving the phenolic OH group of lignin, is responsible for the formation of both copper and copper-ammonia complexes by peat soils.

Second, the reactions of copper and ammonia with aromatic amines, amino acids, thioglycolic acid, and ethyl mercaptan were studied. The data that were obtained indicated that:

1. The groups reacting with copper to form the primary copper-peat complexes are R-SH and R-COOH.
2. The R-SH and R-COOH groups may have to occur in the same molecule to particpate in the formation of these complexes.
3. These complexes contain some secondary or tertiary aromatic amine. The former gives a complex of pCu 6.5 and the latter one of pCu 5.6.
4. The R-NH_2 group, although not a necessary part of the primary copper-peat complexes, is essential for the formation of the secondary copper-ammonia-peat complexes having pCu values of 14.2 and 17.1.
5. The copper-ammonia-peat complex of pCu 17.1 does not necessarily contain an aromatic amine.

Third, seven of these primary complexes, differing in the aromatic amine they contained, were synthesized and purified for chemical analyses for copper, sulfur, and nitrogen. The data that were obtained showed that:

1. These primary copper complexes contained three atoms of copper, two molecules of cysteine, and one aromatic amine group.

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Discussion

Dr. C. R. Dawson: What are the levels of copper concentration in which you measured these potentials?

Dr. J. E. Dawson: Using the copper amalgam electrode, we attempted to determine the range of copper activity over which we could work by determining the solubility products of such compounds as copper sulfide, 10^{-18} molar, and cupric hydroxide, 10^{-64} molar. In this way we measured cupric ion activities over the range of values included in this paper.

Dr. Steward: I have obtained the impression that you have isolated these complexes in the reaction mixture when you have reached a certain pCu value.

Dr. J. E. Dawson: That is correct.

Dr. Steward: Does this correspond to the pK value of the complex?

Dr. J. E. Dawson: No, or at least not necessarily. For example, you will notice that there is one striking difference between the flat portions of these curves in comparison with the type of curve you obtain with an acid-base titration. The acid-base titration normally has a slope of approximately 2 when pH is plotted against degree of saturation; but the slope of these complexes is essentially zero, which is surprising to me. This may be explained by the fact that in the formation of these complexes in the way we carried out the titration, we were forming a saturated solution of the cupric salt of cuprous mercaptide in the early part of the titration and along the flat portion of the curve we were introducing the amine group from a saturated solution of amine. Since the amine was introduced into a constituent with respect to which the solution was saturated, the slope should have remained constant.

Dr. Steward: Wouldn't you expect the complex in the flat portion of your titration curve to consist of a mixture?

Dr. J. E. Dawson: Yes. We did expect a mixture. We didn't isolate it, but we have evidence that it occurred. For example, the copper content of these complexes may be of the order of 30%, but the copper content of the copper mercaptide complex is 44.4%. If you take the precipitate before you reach the flat portion of the titration curve, you approach the value of 44.4%; and if you take it high enough up the curve, it will be 44.4%. If you take the sample when you reach the plateau, you find that it has changed from 44.4% toward the value characteristic of the complex. It was for this reason that a different procedure was used in the actual syntheses.

Dr. Steward: It would seem to me that at the midpoint of the flat portion of the titration curve you should have an equal mixture of the high copper-containing complex and the low copper-containing complex.

Dr. J. E. Dawson: I think you would. We have not investigated this in great detail because of the complexity of the situation and because we changed to another procedure for the synthesis of these complexes.

Dr. STEWARD: One thing I am worried about is the 3 coppers, 2 amino acids, and 1 amine. Is it representative of a single molecular component, or is this an analysis of a mixture?

Dr. J. E. DAWSON: I think it is a single molecular group, as a recurrence group in a polymer, since it was an easily reproducible composition. A molecular weight was not determined because of the insolubility of these complexes. This supports the latter suggestion.

Dr. STEWARD: Wouldn't you expect in sap to have organic acids which would enter into copper-complexing reactions? How would this enter into the picture which you are presenting?

Dr. J. E. DAWSON: The activities of simple acid of complexes would be quite different from the ones we are talking about here. They would be much higher. I think there are many organic complexing reactions which would bind copper, but I believe for the most part they are well out of the range of the complexes we are discussing.

Dr. C. R. DAWSON: What was the activity of your copper-albumen complex?

Dr. J. E. DAWSON: 5.9 in the absence of phenol, and 5.6 in the presence of phenol.

TRACE ELEMENTS AND PHOSPHATE IN HERBAGE PLANT NUTRITION

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The nutrition of herbage plants concerns the transfer of essential elements from the external environment of soil and air to an association of legumes, grasses, and other plants which *in toto* provide the sustenance of herbivora.

The investigations carried out in Australia relating to copper, additional trace elements, and phosphate have differed from most conducted elsewhere, in that herbage plant communities and grazing animals have together constituted a major field of research. The Australian environment has from the initial settlement of the country proved well adapted to pastoral activity and, in particular, to the production of high quality wool. The need for improved animal production and land improvement in regions characterized by liberal rainfall and naturally low soil fertility has brought into relief the relation of these elements to pastures rather than to horticultural or field crops for harvest, which have been largely investigated in other countries.

Deficiencies in Southern Australia

A problem of oats first revealed a trace element deficiency in Australia. Samuel and Piper (41) showed in 1927 that insufficiency of available manganese was responsible for the " grey-speck " disease of this crop in South Australia, and established manganese as an essential element for field crops and herbage plants, including clovers and other legumes. Subsequent experience has proved that the area requiring treatment with manganese is comparatively small and that this element is rarely needed for pasture improvement. A majority of the surface horizons of soil in areas with sufficient rainfall for economic pasture development are characterized by pH values below 6.5, and at which the availability of manganese is high. In fact, negative interactions with manganese are frequent and manganese toxicity characterizes some areas.

It was established in 1937 by Riceman, Donald, and Piper (35) that cereal grains and herbage plants respond to copper on a calcareous sand. Wheat, oats, and barley were improved considerably in grain production by dressings of copper sulphate. *Medicago sativa* (lucerne or alfalfa) and *Trifolium subterraneum* (subterranean clover), the principal cultivated herbage legumes of southern Australia, have since been found to respond to copper on a variety of soil types.

A review was made by Prescott (31) in 1938 of the work carried out in Australia and New Zealand at that time on deficiencies of the soil as revealed by low yields, low carrying capacity for stock, and by so-called plant and animal diseases. Mutual relationships of phosphorus and nitrogen, together with the discovery of the importance of zinc, copper, and manganese in plant nutrition, were indicated.

The significance of zinc in the nutrition of herbage legumes, especially *Medicago truncatula* * (barrel medic), was revealed by Riceman and Anderson in 1941 (34), and confirmed in numerous experiments with subterranean clover subsequently (13, 33, 48, 51, 54).

Investigations over 5 years at one specific centre culminated in the demonstration by Anderson in 1942 that improved development of clover after burning was due to molybdenum in the ash (2); and this element was shown to be essential for nitrogen fixation in subterranean clover and other legumes (7).

These trace element effects are closely dependent on that of phosphate, which has been known to be acutely deficient in the soils of southern Australia for more than half a century. Phosphate, like manganese and copper, was first employed for cereal crops, mainly wheat; but its value became greatly emphasized by the spontaneous development of herbage legumes, especially *Medicago denticulata* (burr medic), in cereal stuble following its use with the preceding crop. The use of subterranean clover in southern Australia and New Zealand began actively to expand when it became realized that applications of superphosphate were needed to ensure high productivity, active nitrogen fixation, and improved soil fertility through this clover. Trumble and Donald (50) emphasized the relation between phosphate dressing, nitrogen fixation, and the subsequent development of grass dependent on nitrogen gained by the root nodule organism (*Rhizobium trifolii*). The investigations of more recent years (3, 6, 10, 33, 47, 48, 51, 53, 54) have indicated widespread trace element and phosphate deficiency in southern Australia. Frequency of interaction between nutrients and a close dependence of nutrient uptake on environmental influences are characteristic features.

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* syn. *M. tribuloides.*

Copper has proved one of several necessary keys to the increased production of plant protein and improved fertility of the soil under conditions previously considered incapable of supporting agriculture. The observed effects of copper, however, are closely dependent on mutually operative factors; and it is the purpose of this paper to indicate some of them. The nutritional complex of a herbage plant association is governed by a multiplicity of interactions which differ greatly in pattern according to environment but are likely to reveal consistencies when examined in terms of common units.

Sometimes the arrangements of such patterns resist interpretation because some of the units have been imperfectly defined for the purpose in view. Those of climate, while strongly influential, have yielded little precise information on variables which govern the assimilation of copper and other nutrients because of the inadequate measures of climate that have been employed.

GEOCHEMICAL INFLUENCES

The weathering processes leading to soil formation, the degree of leaching or deposition, availability of nutrients according to pH, characteristics of the clay and organic matter, together with the effects of actively growing plant roots, bear closely upon the problem of nutrient uptake; but the geochemical composition of the material from which the soil types concerned have been derived is a logical first consideration in the location of soil deficiencies (Fig. 1). Piper

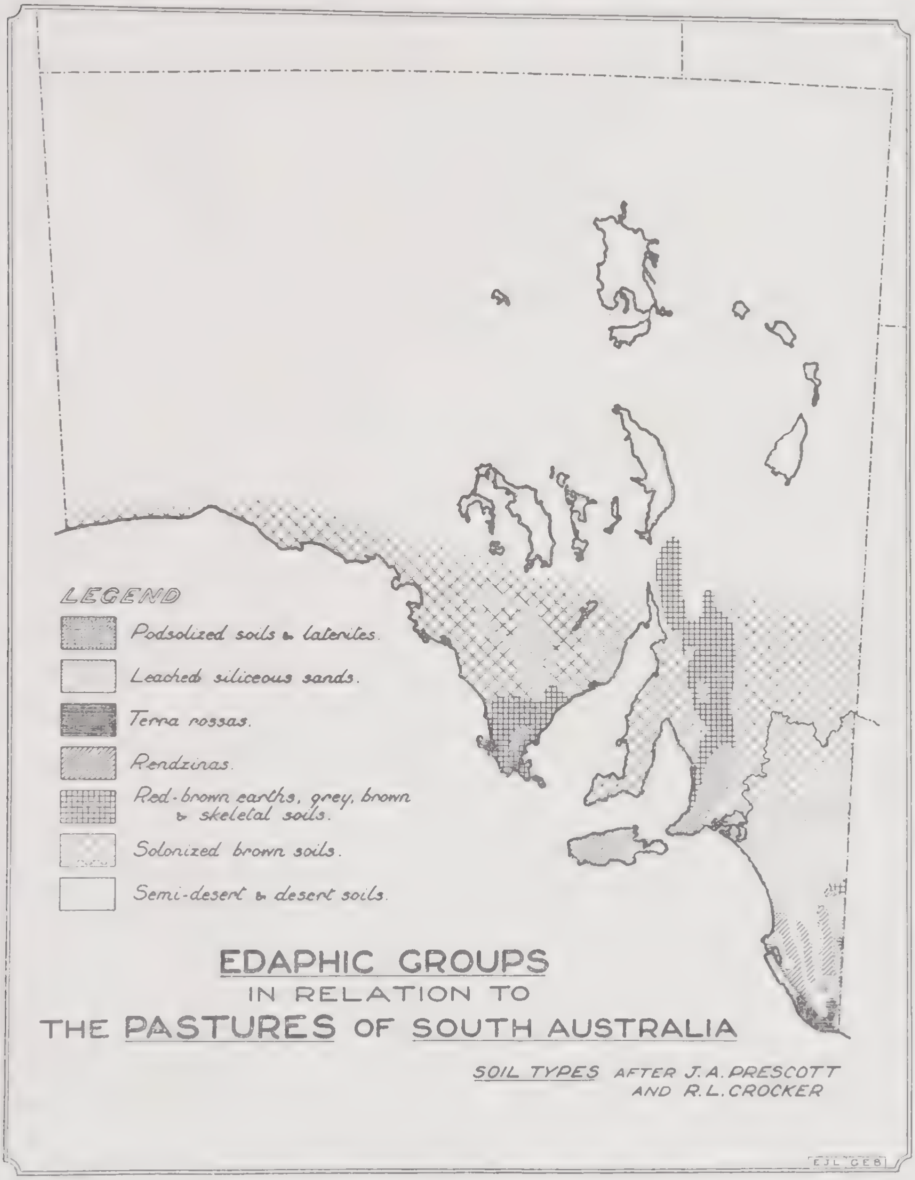

LEGEND
Podsolized soils & laterites.
Leached siliceous sands.
Terra rossas.
Rendzinas.
Red-brown earths, grey, brown & skeletal soils.
Solonized brown soils.
Semi-desert & desert soils.
EDAPHIC GROUPS
IN RELATION TO
THE PASTURES OF SOUTH AUSTRALIA
SOIL TYPES AFTER J. A. PRESCOTT AND R. L. CROCKER
EJL CEB

FIG. 1. Edaphic Groups in Relation to the Pastures of South Australia.

and Prescott (29), referring to the principles laid down by Goldschmidt (14) and the reviews by Wallace (55) and by Mitchell (23) concerning geochemical factors, indicate some of the generalizations that apply to Australian soil problems. These include the dependence of isomorphous replacement on the ionic radius and ionic potential of specific elements, the early crystallization of phosphorus from magmas as apatite together with ilmenite and magnetite, the greater abundance of zinc and copper in basic than in acidic rocks, and the concentration of molybdenum in silica-rich magmas. The importance of geochemical factors in problems of soil fertility in Australia was originally stressed by Thomas (46).

Little is known concerning the fate of trace elements during successive cycles of weathering. Phosphate is not randomly distributed in plutonic rocks and occurs multimodally in South Australian soils (29, loc. cit.). Oertel and Prescott (25) have shown that the formation of concretionary iron oxide in laterite immobilizes phosphorus, molybdenum, zinc, and other elements as anionic oxides. Soil surveys conducted in Australia over the past 25 years have revealed that a large proportion of the Australian land surface is covered by what are virtually fossil soils and their later derivatives (30, 43). The influences of podsolization and solonization superimposed upon highly calcareous parent material also account for some fairly widespread occurrences of phosphate and trace element deficiency; and current climatic influences are important.

The status of total phosphorus is generally low in Australia. Crop production and pasture improvement are not usually undertaken in the south without the application of superphosphate.

Total copper is extremely low in calcareous and siliceous sands on which the most widespread cases of copper deficiency occur. The accumulation of calcareous sands from foraminiferous shell have characterized the coastal areas of southern Australia since the Pleistocene. Uplift has placed some of the resulting dunes inland. Further from the coast are solonized brown soils which are also highly calcareous but associated with lower rainfall. These are considered by Crocker (9) to have been formed from loess derived by the winnowing of deposits exposed by uplift above sea level. Both groups tend to be low in copper and zinc.

Solonized sands of high silica content and largely of associated fluviatile and aeolian origin occur widely and are also extremely low in phosphate, copper, and zinc.

Molybdenum deficiency has been associated for the most part with much older rocks or with lateritic formations high in iron and aluminum. Prescott recognized laterite in Australia as a fossil soil horizon gentically related to the podsolized types (30), and the lateritic remnants now evident are considered to be residuals following elevation and dissection in Pleistocene and Recent times (43). The impoverishment of these soils in phosphate, molybdenum, and zinc is due not only to leaching of the profile and the concretions already mentioned, but also to the quality of the original parent materials.

SOIL FACTORS

It has been pointed out by Piper (26, 27) that extremely low concentrations of copper, zinc, and molybdenum will satisfy the needs of herbage plants provided these concentrations are maintained.

Plants secure sufficient copper if the element remains available at 0.1 parts per million. Concentrations as low as .001 p.p.m. or 1 μg. per liter are capable of producing some increase in growth, which can be directly correlated with the level of copper between .01 and .001 p.p.m. Copper may exchange freely with other exchangeable cations in the soil. The ionic radius of copper (0.80Å) is sufficiently close to that of zinc and ferrous iron (0.83Å) to permit entry into a common crystal lattice. Soil reaction has some effect on availability, which tends to decrease with increasing pH, but this element is less susceptible to changes of pH than zinc, and is very much less susceptible than manganese or molybdenum.

Zinc can also satisfy the needs of the plants at extremely low concentrations provided absorption is continuous and utilization by the plant effective. The solubility of zinc decreases as the pH of the soil rises from 5.6 to 7.5, above which concentrations are at a minimum. Walkley, quoted by Piper (27, loc. cit.) found 0.1 to 0.3 p.p.m. of zinc remained in solution, even at minimum solubilities, and these if maintained, are sufficient for the needs of growing plants.

Molybdenum occurs in available form mainly as molybdate. It is the only trace element found necessary in southern Australia that becomes increasingly available with increasing pH. Piper and Beckwith (28) have shown that the concentration of molybdenum in the dry material of *Medicago denticulata* increased from 0.2 p.p.m. at pH 4.8 to 6.5 p.p.m. at pH 8.3 in the absence of applied molybdenum; where 2 lbs. per acre of molybdate were applied, the concentrations were 5 p.p.m. at pH 4.8 and 56 p.p.m. at pH 8.3.

South Australian soils are extremely low in total phosphate. The lighter types frequently contain less than 100 p.p.m. of acid-soluble PO_4 (29); annual applications of superphosphate at rates of 20 to 50 lbs. of P_2O_5 are regarded as essential for all pasture improvement based on herbage legumes. The nitrogen gained by subterranean clover rises considerably with increasing phosphate application, and the amount accumulated by the root nodule organism determines the subsequent development of pasture grasses.

Symbiotic nitrogen fixation appears to be capable of supplying the nitrogen requirements of subterranean clover and associated grasses in South Australia (52), provided the supply of available nutrients and their balance is satisfactory and restrictive influences are not limiting.

It has been the fate of laterized and podsolized soils of sufficiently low pH, with sloping topography, to become deficient in available molybdenum following some years of active clover production, under dressings of superphosphate alone.

Negative interactions between manganese and other trace elements are frequent on acid soils. In view of the opposite effects of varying pH on manganese and molybdenum, wide departures from neutrality could produce highly unfavorable ratios of these two elements.

Leaching and water logging, in addition to influencing availability of plant nutrients, govern the efficiency of nitrogen fixation by legumes. Concentrations of soluble salts and organic breakdown products are controlled by water movement and the degree to which drainage or deposition occurs. Toxic concentrations are capable of inhibiting the effects of nutrients which occur at favorable levels.

Herbage plants have responded to phosphate, copper, zinc, and molybdenum on heavy clay irrigated flats in South Australia after reduction of surface salt from above 0.4 per cent to 0.05 per cent by leaching (54). On wet heath soils in the southern part of South Australia leaching or oxidation is necessary before subterranean clover will respond effectively to potash, phosphate, copper, zinc, and molybdenum on these soils (11). The leachates are organic in character and depress subterranean clover when applied to fertile soils.

Recent observations in Nicaragua have revealed that *Acacia cookii* completely inhibits the growth of all plants, including grasses, within the root zone (32). The root excretions of this legume warrant investigation.

Depression of subterranean clover in South Australia by leachates is due to inhibition of the root nodule bacterium and not the host plant. Deficiencies of essential elements in the plant are known to produce characteristic changes in organic acid content (16); and it would be surprising if natural vegetation on geochemically deficient soils did not lead to toxicity based on the accumulation of such products under conditions of reduction.

The influence of soil microorganisms on the availability of plant nutrients has been little investigated. Their effects are doubtless extensive. Forms acting on cellulose or lignin may release phosphate and trace elements from both minerals and soil organic matter. On the other hand, certain forms may compete with the growing plant and immobilize phosphate and zinc. The nature of both the organic matter and the microbial population of soils varies widely. Deficient soils appear to contain considerably more organic matter in a relatively undecomposed state.

Organic matter in quantity may not necessarily indicate high soil productivity. Copper deficiency was first recognized on peat soils and in southern Australia copper deficiency occurs on soils of widely varying organic content. If the parent rock is low in one or more trace elements, it is reasonable to expect natural vegetation to reflect the status of the elements concerned.

Climatic Effects

The nutrition of herbage plants is closely modified by climatic changes. The influence of temperature and light on photosynthesis, their direct effects on the growth rate (21), and the dependence of respiration on temperature all lead inevitably to shifts of carbohydrate status and energy potential which may govern nutrient uptake, subsequent growth, and the appearance of deficiency symptoms. Variations of soil moisture content, through their effects on solubility and the relative concentrations of different ions, may also closely modify nutrition. Concentrations of nutrient elements vary widely from season to season despite uniformity of plant material, physiological growth stage, and the conditions of soil apart from changes in soil moisture.

The differences in the chemical composition of plants grown in tropical and temperate regions are well known. Under conditions of high temperature and abundant moisture, a high rate of photosynthesis can be expected to produce herbage with a lower content of protein and mineral elements more rapidly than at lower temperatures.

The metabolism of herbage legumes is of critical significance during the first two or three months of development from the seed, rhizome, or rootstock. If nutrient uptake is adequate at this stage, the basis is laid exponentially for high productivity as physiological maturity advances. If on the other hand, deficiency or toxicity becomes evident early, there is little chance of high production later.

Zinc deficiency of corn, tomatoes, and citrus has proved most acute at relatively high temperatures and high light intensity in California (18) and has been overcome by appropriate shading. Reverse effects are found in South Australia with winter-grown long-day plants such as subterranean clover and flax. Deficiency symptoms of phosphate, zinc, and molybdenum are acute in winter; symptoms of copper deficiency are more evident later, although the restriction of seed production, where available copper is lacking, results in low populations from the onset of the early winter rains.

Investigation by Ferres (12) showed that *Trifolium pratense* (red clover) behaved at Adelaide in the opposite manner to subterranean clover, or similarly to summer-grown annuals and citrus in California. The two herbage legumes synthesize carbohydrate in the early stages under reverse changes of temperature and light. Red clover commences at a day length of approximately 12 hours, rapidly increasing to more than 14 hours in southern Australia. Subterranean clover commences at a day length of slightly less than 12, soon decreasing to 10 or less hours, at the same latitudes. The critical period at which zinc deficiency appears is therefore associated with light duration extending above 12 hours in the first case and contracting below 12 hours in the second. The uptake of zinc by subterranean clover is substantially increased by counteracting reduced day length and temperature, while a similar effect is produced in red clover by counteracting the opposite trend. Treatment in each case modifies the conditions of light and temperature towards a common set of values, approaching 12 hours of light duration and mean air temperatures in the neighbourhood of 60°F. Subterranean clover is well adapted during its early vegetative growth to this combination of values, and the same may be true of numerous other herbage legumes.

Climatic studies of herbage plant reactions indicate that the yield and composition of a pasture tends to be closely controlled by favorable combinations of such factors at an early developmental stage. A recent examination of variations in annual yields of coffee in Nicaragua (32) provides a cogent example of this principle. The study showed that yield was dependent on the departure of the rainfall in May, in the month of flowering, from a mean of 8.73″. Each inch above or below this value produced a decrease of 60 lbs. of coffee per acre, or 8 per cent of the possible yield. An excessively wet month, or an excessively dry month, could reduce the yield by at least 50 per cent. Assuming that subsequent development of the coffee berry is a highly catalytic process, the shift in the requirement and fate of copper or any other trace element concerned must vary greatly from season to season; and the metabolism of micronutrient in relation to the nutrition of the entire plant can only be studied effectively after such predetermining factors have been taken into account.

The herbage association, although more complex, is similarly controlled; and the integrated climatic pattern over the period of seasonal growth is subsequently reflected in competition among components for nutrients, moisture, and light, and is affected by the imposition of the pruning influence of the grazing animal or the mechanical mower.

Seasonal Patterns

Attempts to interpret the exchanges concerned in herbage plant nutrition are not likely to be productive in the absence of a realistic picture of the climatic changes that influence nutrient uptake, translocation, synthesis, and the growth of the plant. It has not been orthodox to define simultaneously the sequential changes of day and night temperature, light duration and intensity, and the level of available soil moisture over the growth period, but this objective is both attainable and sound in principle.

It is usual to accompany reports of field investigations with some description of climate, but the inadequacy of the climatic data presented is widely recognized. There are two reasons for this. First, there is insufficient appreciation of the profound effects of a few relatively simple but constantly varying factors. Secondly, it is only rarely that the climatic variables of greatest consequence are appropriately measured (49).

It seems desirable to approach the problem by confining first considerations to the primary factors of moisture, temperature, and light. The supply of moisture to the growing plant is governed by precipitation and irrigation on the one hand, and by run-off, percolation, evaporation, and transpiration, on the other. The balance of moisture gain and loss can be traced from suitable data on rates of precipitation and evaporation in relation to field capacity, wilting point, and growth of plant, and checked in practice by the continuous determination of soil moisture in terms of electrical resistance.

Indices to the temperatures of transpiring leaves and absorbing roots may be derived from the daily measures of atmospheric shade, temperature, and humidity. These values and their daily fluctuations can be used with considerable effect if variations from standards according to both time and micro-environmental location are appreciated and understood.

Light involves both duration and intensity. Quality, while not unimportant, does not appear to offer such a fruitful field for investigation; most artificial radiations that can be employed result in abnormal effects on the plant. It seems justifiable to accept the normal range of variation in daily light quality, assuming physiological adaptation by the plant through compensatory effects. Single measures of solar radiation can be applied to evaporation or photosynthesis; but in nutritional studies, it is desirable to separate temperature from light, and transpiration from other sources of moisture loss. Continuous measures of light intensity within the range 0-1000 f.c.p. would be extremely valuable in studies of plant nutrition according to climate, but are almost completely lacking in standard meteorological data.

A simple, integrated method of climatic representation has been recently devised (48) to illustrate the relation of herbage production to climatic variation over 24 seasons at Adelaide. No two years have the same climatic pattern and no season is duplicated by the mean. This approach could be used with considerable effect in nutritional studies (Fig. 2). Its basis is the simultaneous expression of light duration, temeprature, and moisture. Amplitudes of variation in temperature according to day and night can be added, and changes in light intensity superimposed. Irrigation needs, and excess moisture, are evident. The time interval so far adopted is one calendar month; but obviously the blocks can be divided into shorter periods of any chosen length.

The aim is to seek major classifications of integrated values for energy potential and moisture. Limiting combinations of factors are likely to prove critically operative for relatively short periods of time. The definition of physiologically acceptable parameters should render it possible to subclassify and relate indefinitely. The immediate objectives are to provide an introductory picture of the concurrent climatic changes to which growing plants are subject. These closely govern nutrient uptake and use, but have so far been little investigated as determining factors in plant nutrition.

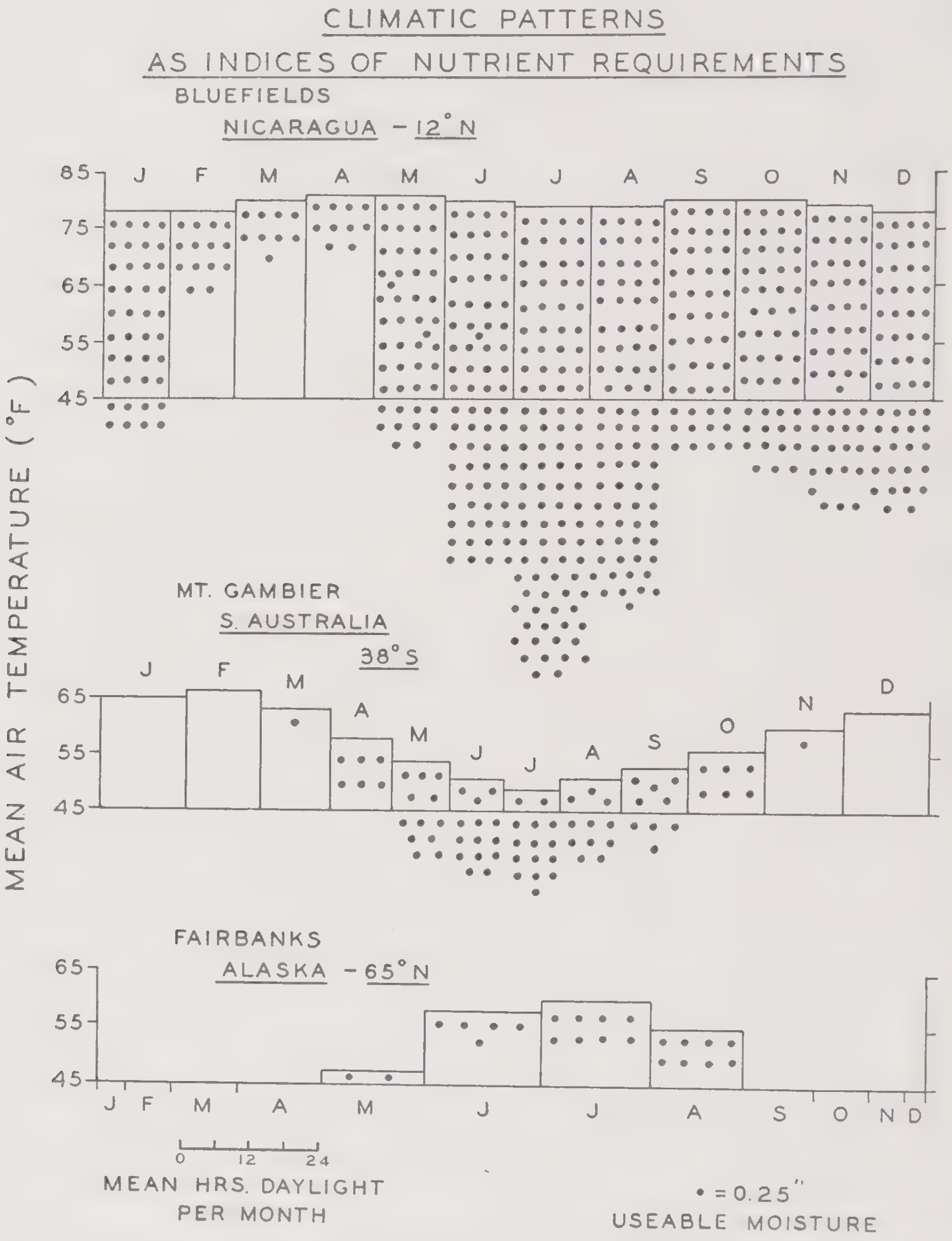


FIG. 2. Climatic Patterns as Indices of Nutrient Requirements
Ordinates, mean air temperatures. Abscissas, months scaled to mean hours of daylight per month. Dots, above baseline, 0.25 in. of useable moisture; below baseline, 0.25 in. of moisture unuseable because temperature or daylight are limiting factors. Blocks, amount of moisture available to plants for growth under each particular month's average temperature and daylight.

NUTRITION OF HERBAGE LEGUMES

Herbage legumes tend to be suppressed by associated gramineous plants, unless the latter are reduced by low available nitrogen in the soil, or by close defoliation. The nitrogen poverty of most unproductive soils ensures a low plane of competition at the outset. As nitrogen is gained through symbiotic fixation and urinary excretion, grasses increase in vigor, but may be controlled under grazing or mowing, to maintain a legume complement (Fig. 3A).

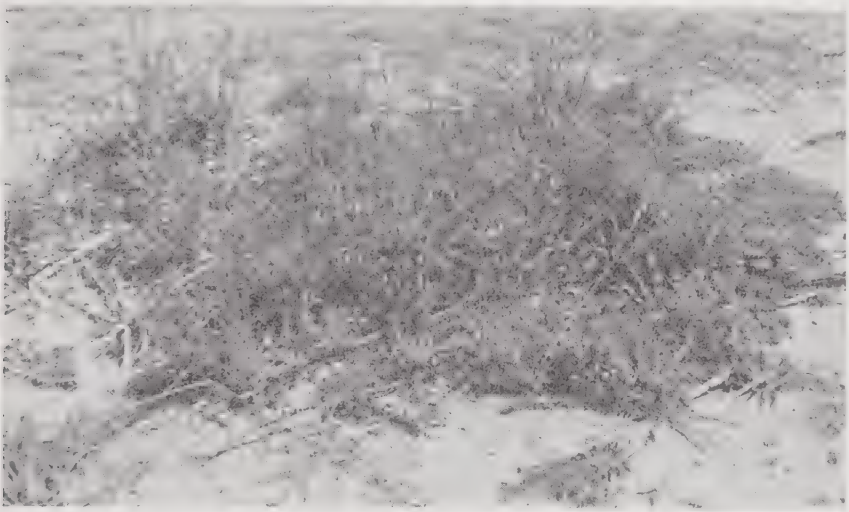


FIG. 3. Responses of Subterranean Clover to Micronutrients

A, Development of siliceous sands through subterranean clover, lucerne, *Ehrharta*, and added phosphate, copper, and zinc. Second-year pasture of clover, lucerne, and associated grass after first-year treatment with 40 lbs. P_2O_5, 7 lbs, $CuSO_4$, and 14 lbs. $ZnSO_4$ per acre.

Apart from mineral nutrients, herbage legumes require a favorable balance of moisture and air in the soil, together with conditions of temperature and light within an optimal range according to growth stage, in order to fix nitrogen actively. Most legumes are sensitive to pH of the soil; but it is not known to what extent this operates through nutrient availability, or infection by *Rhizobium*. It is of practical moment in southern Australia that *Trifolium subterraneum* tends to be inhibited by pH values exceeding 7.0, whereas

Medicago spp. are most productive above neutrality and disappear at acid reactions. *T. subterraneum* and *M. truncatula* between them are capable of fixing nitrogen actively at pH values from 5.0 to 9.5, but because of their distinct requirements are rarely found growing in association. Both are closely dependent on phosphate, copper, zinc, and molybdenum for high nitrogen accumulation.

Phosphorus is concerned primarily with the growth of the host plant, especially in the early developmental stages. Application of phosphate to phosphate-deficient soil increases the concentration of phosphorus within the plant, except under relatively dry conditions, when it is depressed (39). It also reduces the rate of transpiration per unit of leaf surface, and the leaf-weight ratio, thus leading to a marked reduction in the transpiration ratio (58). As a result of recent experiments, Bonner (8) has considered indoleacetic acid to be associated with phosphorus metabolism, and the utilization of respiratory energy in the plant cell to be related to the formation and transfer of high energy phosphate.

Copper affects the seed production of annual legumes, but appears also to influence the later development of perennials, including the accumulation of reserve food material, and thus relates to the persistence both of annual and perennial species. Deficiency of copper is rarely acute enough in practice to inhibit the early vegetative growth of subterranean clover, but the leaf, stem, and root development of lucerne (alfalfa) is frequently improved by the addition of copper to soils associated with calcareous material.

Zinc is concerned primarily with early vegetative growth. It affects both the relative growth rate and the net assimilation rate of subterranean clover (12). Zinc uptake is influenced by air temperature rather than soil temperature, and by light duration, which also affects the distribution of zinc in the plant. Deficiency symptoms are most severe in the early leaves, but have not been observed in clover until after the first trifoliate leaf.

Molybdenum is concerned primarily in symbiotic nitrogen fixation. Direct effects on the host legume are slight by comparison (Fig. 3B, C). Jensen (19) has calculated that 10 to 25 p.p.m. of Mo in the nodule are required for maximum nitrogen fixation. The rate of fixation decreases with reduction below 10 p.p.m. The plant appears to have a wide tolerance of molybdenum concentration; adverse effects on grazing animals become evident with concentrations above approximately 10 p.p.m. associated with suboptimal copper. The ratio of copper to molybdenum in herbage, as it might affect animal health, is currently receiving detailed attention.

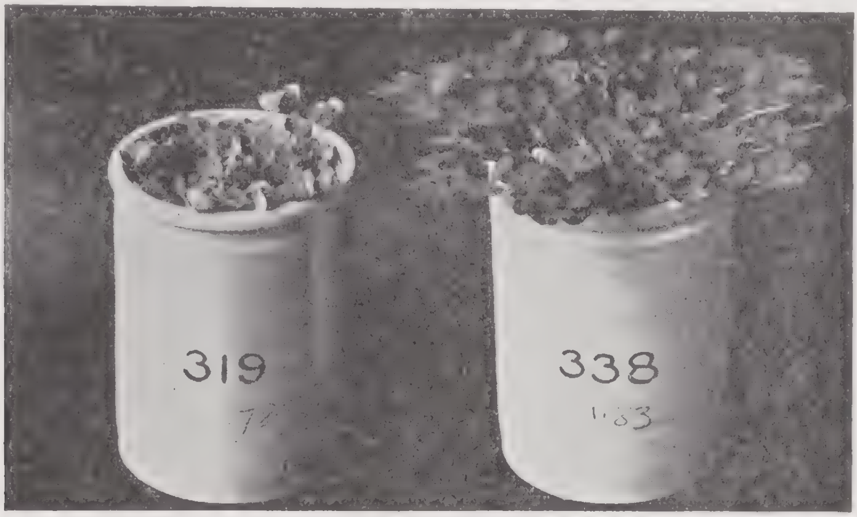


FIG. 3. B, Response of subterranean clover to molybdenum in pot cultures of 1 kg. capacity, 12 weeks from seeding.

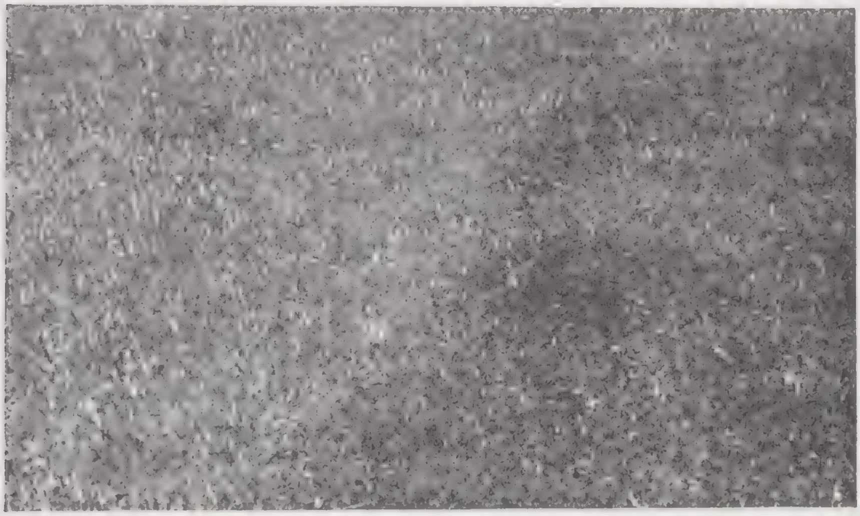


FIG. 3. C, Response of subterranean clover to molybdenum in the field, under grazing conditions, in winter. Left, added superphosphate. Right, added superphosphate plus ammonium molybdate, 2 oz. per acre.

Boron deficiency primarily restricts nodulation and nitrogen fixation, but may also, under certain conditions, limit the growth of the host legume directly. Neither boron nor manganese has been found deficient in subterranean clover pastures, and the use of manganese for herbage, even on calcareous soils, is limited. Anderson and Spencer, quoted by Anderson (4), have shown that manganese sulfate may decrease symbiotic nitrogen fixation by reducing molybdenum uptake.

The presence of hemoglobin in healthy nodules has tended to emphasize oxygen status in symbiotic nitrogen fixation, which is inhibited when the red pigment is absent. Breakdown of hemoglobin leads to formation of methemoglobin and development of a green coloration of the nodule. This is a feature of plants that fail to fix nitrogen, when grown on new lands characterized by multiple deficiencies and toxic derivatives (11). The low oxygen status of the nodule suggests that hemoglobin may here combine with molecular oxygen as in blood. Hill (17) has pointed out the similarities of the structure of chlorophyll and the Fe porphyrin of hemoglobin, and indicated that increasing individual amounts of the three metals, Cu, Fe, Mg, may produce a series of interdependent catalytic reactions in the development of the photosynthetic system of the green leaf. Such reactions may also concern the effectiveness of the root nodule of the herbage legume. Although the function of molybdenum in reducing nitrate to ammonia is generally recognized, Meijering and Rathenau (22) have shown that molybdenum trioxide greatly accelerates the oxidation of iron base and copper base alloys. They consider that the appearance of a liquid oxide phase is the determining factor and not the dissociation of molybdenum trioxide.

Iron and magnesium both appear to be present in sufficient amounts for herbage plant nutrition in the Australian and New Zealand soils so far investigated. Calcium and sulfur, both of which are closely concerned with nodule formation and the internal metabolism of the host plant, are applied automatically with superphosphate to improved pastures in these two countries.

Anderson (4) has pointed out that legumes deficient in nitrogen, molybdenum, zinc, or phosphorus tend to be well nodulated, but that poor nodulation usually results from low calcium or sulfur. It has been suggested that sulfur deficiency may decrease symbiotic nitrogen fixation in part through its effect on nitrogen metabolism within the plant (6).

Potash is concerned in the translocation of carbohydrate. Positive interactions with molybdenum are frequent where the supply of available potash is limited; but the needs of annual legumes can apparently be supplied from the subsoil through associated deep-rooted pasture grasses.

After deficiencies have been overcome by initial fertilizer treatment, applied nutrients, other than phosphate, and possibly zinc, appear to be sufficiently available for some years, at least under grazing. Unfavorable conditions of balance may develop, however, and periodic cultivation is required to facilitate oxidation and the release of nutrients from organic matter which accumulates in unploughed grassland.

SOIL-PLANT RELATIONSHIPS

Comparatively little work has been carried out on soil-plant relationships as they affect the availability of phosphate and trace elements. It is known that different species or varieties of the same species vary in their capacity to absorb relatively unavailable cations or anions (35, 36, 39). It has also been shown that plants vary during the growing season in capacity for nutrient assimilation. Ferres (12, loc. cit.) has shown, for example, that zinc is taken up in higher concentration with increasing photosynthesis. This also appears to be the case with phosphorus and copper. Energy status affects the movement of water through and to the plant, root respiration, and microenvironmental changes of pH and pO_2 in the immediate vicinity of absorbing roots, thus influencing nutrient exchange.

Jenny and Overstreet (20) have indicated that depletion of adsorbed cations through substitution with hydrogen in the solid phase is one main result of the merging of the electrical layers of the colloidal systems of plant root and soil particles in contact exchange. The soil, plant, and atmosphere form a dynamic interdependent system and the loss of both organic and inorganic ions by the root may affect intake.

Richardson, Trumble, and Shapter (40) showed that potash could be lost in quantity from grass roots during senescence. The conclusions of Virtanen concerning the excretion of amino nitrogen from legumes stimulated considerable work in Australia on associated growth. This showed that such excretion was not a normal feature but could be induced under somewhat unusual conditions of climate (45, 52). Increasing concentrations of soluble nitrogen may occur in the plant through reduced photosynthesis and increased transpiration.

Epstein, quoted by Stout (44), has concluded from experiments with growing plants and clay suspensions involving use of radioactive isotopes of high specific activity that the principal factor determining uptake is the degree of saturation of clay with respect to adsorbed micronutrients, and that different proportions of the complementary ions Ca and H were of less importance. Stout also reports that many soils fix zinc in non-exchangeable form, and that this is particularly so in material of a micaceous character.

Gregory and Sen (16) and Richards (37) considered that the efficiency of respiration tends to be optimal when the plant is growing under " balanced " conditions of nutrition. There are arguments for considering such balances as elastic forms of gene-nutrient-energy-moisture relationship, within which considerable latitude of combination and tolerance provide for a given yield and composition. Measures of specific nutrient factors could with advantage be expressed as departures, whether positive or negative, beyond tolerance limits, as well as in terms of absolute values.

Under favorable conditions of moisture supply and temperature, the yield of subterranean clover, on a podsolized sand containing less than 5 lbs. of available P_2O_5 and 10 to 15 lbs. of available N per acre, followed the well-known Mitscherlich relationship in each of three seasons, with increasing applications of superphosphate, to a maximum of 94 lbs. of P_2O_5 in any one year (50). Phosphate recovery in the herbage was directly proportional to the dressing of phosphate, to a limit of 141 lbs. of P_2O_5 per acre applied over the three seasons. Recovery was 20 to 40 per cent of the phosphate applied. The percentage of nitrogen in the clover rose with increasing phosphate application, and the subsequent development of grass was determined by the nitrogen previously fixed.

Interactions between phosphate and molybdenum have been well shown by Anderson and Oertel (5). Those of potash with molybdenum are frequent when the roots of clover plants are restricted to the surface soil horizons only (51).

Numerous interactions of nutrient effects have been recorded but are still not understood. The factorial experimental design has proved invaluable in establishing the degree of the responses obtained from applications of nutrients, singly and in groups; but more advanced physiological studies should accompany field experimental work. Increasing yield according to treatment is expressed by the Mitscherlich relationship where only a single limiting factor is involved, as in the experiment with phosphate quoted above. But as shown by Gregory (15), single factor relationships are the exception, and the operation of several factors simultaneously, the usual condition. The resistance formula of Maskell

$$\left(1/y = \frac{C(a)}{a_1 + a_2} + \frac{C(b)}{b_1 + b_2} + K\right) \qquad \text{where } y = \text{the yield,}$$

C(a) and C(b) are constants characteristic of nutrients (a) and (b) respectively, a_1 and b_2 are the amounts of a and b that can be absorbed from untreated soil and a_2 and b_2 the amounts of these nutrients applied, and K is a constant covering all other factors, outside nutrients a and b which are maintained at constant operative effect, is recommended by Gregory (15), who has presented evidence for its value as a method of representation. Such a formula provides not only a measure of the efficiency of each fertilizer element involved, but also an estimate of availability of the nutrient already present in the soil. Both the Mitscherlich and the resistance formula depend on curvilinear relationships. Liebig's original law of the minimum is of the linear type.

The type of yield curve secured will depend on the degree to which positive or antagonistic interactions between different elements occur within the plant. Antagonism tends to produce modifications from the standard autocatalytic form towards a sigmoid type of relationship (56). The diagrammatic methods of Nicholas (24) demonstrate antagonistic and mutually beneficial effects between elements. These effects can be shown radially or by means of histograms. The method of foliar diagnosis has proved especially useful in assessing the nutrient balance of the plant at different stages and under varying conditions of treatment. Its combination with factorial field plot and pot culture trials can effectively establish the nature of the interactions concerned; but subsequent biochemical and physiological examination, closely orientated to the conditions of the field, is needed to permit the development of interpretations that may be fairly generally applied. The use of radioactive isotopes can prove exceptionally informative provided the controlling variables of climate are sensibly imposed upon the soil plant relationships studied.

Many cases of trace element deficiencies have been overlooked through the emphasis placed on obvious deficiency symptoms, either plant or animal. It is becoming increasingly appreciated that numerous cases of underproduction are associated with suboptimal but not acutely deficient levels of a particular nutrient or nutrients, or to marked imbalance. Such conditions are difficult to diagnose and can only be discovered by actual field experiment. The animal may respond to concentrations above those needed by the plant; pastures in Western Australia respond to zinc containing 10 to 15 p.p.m. of Zn on a dry basis, which is more than sufficient for animal requirements (53).

Sheldon, Blue, and Albrecht (42) have shown that concentrations of the amino acids in the plant can be modified by trace element treatment and that certain effects of commercial fertilizers are due to trace element impurities. This effect of impurities was shown by Trumble and Ferres (51) to be considerable in the case of relatively impure wartime phosphate.

DEVELOPMENT OF NEW LANDS

Man has for many centuries selected those soils most capable of providing de novo the nutritional needs of crops; and it is well known that the food supplies of the world are grown on only a small portion of the total area with a favorable climate. The growing knowledge of herbage plant nutrition opens up attractive possibilities for developing naturally unproductive lands.

Little attention has been given to the ash content of indigenous vegetation and to the manner in which this has accumulated over many years from different soil horizons. Molybdenum deficiency was detected in South Australia following the observation that the burning of tree stumps and logs led to a much improved growth of subterranean clover. Such effects have long been recognized. The influences of high temperatures on soil, due to partial sterilization or liberation of an essential mineral in available form, as for example, release of copper in calcareous sand by heating (27), are fairly well known. Until the last decade, the main effects of wood ash on plant production were attributed to potash. After it had been shown that wood ash was responsible for burning effects on laterized soil, potassium and copper were considered as possible effective constituents. The soil responded to neither, alone or in combination with any other nutrient except molybdenum. The full effect of 5 tons of wood ashes per acre was obtained with a dressing of 1 oz. molybdate or molybdenum trioxide per acre (2, 5).

Absence of response to trace elements on newly developed land may be due to their presence in the ash or organic residues of the natural vegetation, to a primary deficiency of phosphate and nitrogen, or to adverse soil factors, in addition to their presence in the soil at favorable concentrations. Warren and Howatson (57) have suggested that the zinc and copper content of some trees reflect concentrations of zinc and copper in underlying soils and rock formations. Certain species have been listed as offering possibilities for detecting unusual or abnormal concentrations of these elements. Wood contains the least and leaves the greatest concentrations. In the samples examined, zinc appeared on the average to be twice as abundant as copper.

Deep ploughing, to destroy all native vegetation after burning, is now standard treatment in South Australia. This mixes the surface layers of soil to 10 inches or more. The effectiveness of burning before ploughing governs both initial trace element concentrations and the degree to which toxic residues remain. Extracts from the leaves of *Banksia* depress *Rhizobium* to a greater extent than from other parts of the plant.

The use of a preliminary crop assists in the early absorption of adverse concentrations and helps to provide more readily destructible organic matter. Evidence has been available for some years that phosphate, copper, and molybdenum may produce effects a season or more after application. These effects are largely interpreted as microbiological. Trumble, Donald, and Strong (50) found an increased total soil bacteria count from 38 million to 400 million per gram of dry soil as a result of phosphate application. The known effects of trace elements on microfungi and bacteria suggest that numerous functional relationships await investigation.

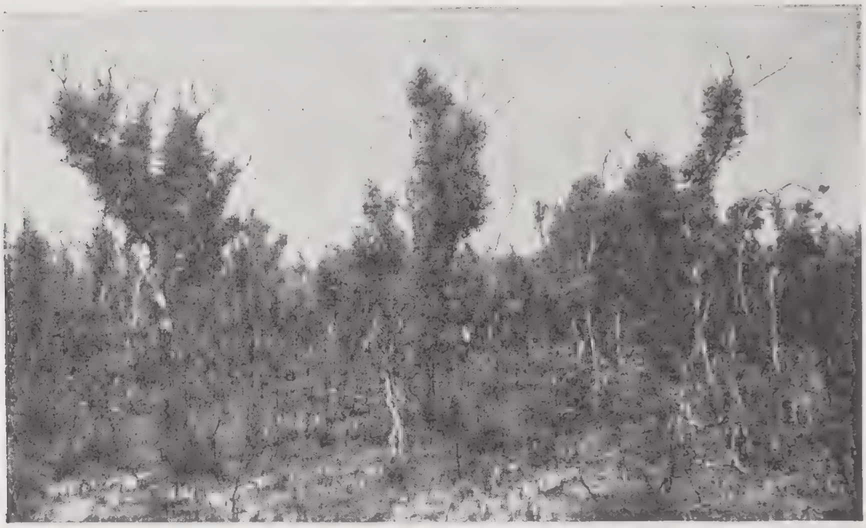
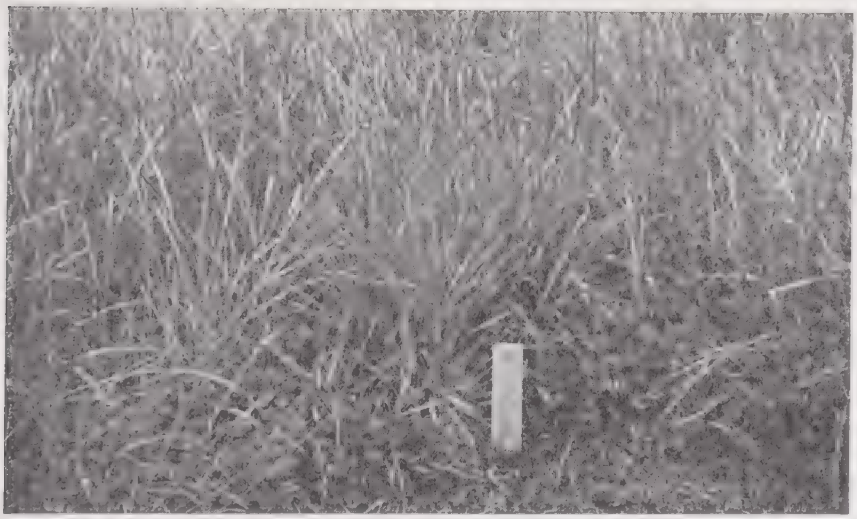


FIG. 4. Development of Podsolized Sands through Subterranean Clover, and Added Phosphate and Molybdenum

A, Sclerophyll forest, partially burnt, prior to clearing.

The maintenance of sheep and cattle on newly developed pastures, which are first legume dominant, but become higher in grass as nitrogen enrichment proceeds, now characterizes much naturally unproductive country in southern Australia (Fig. 4). The pastures continue to improve with time, and the practice has proved economically successful.

B, First-year development of clover receiving 80 lbs. P_2O_5 per acre. Molybdenum, initially available from burning the native vegetation, subsequently becomes deficient.



C, Third-year development of seeded grass, nitrogen of which has been provided in toto through symbiotic fixation in the clover.

Phosphate fertilization to an accumulated amount of 120 lbs. of P_2O_5 over a 3-year period appears to have established a sufficient reserve of available soil phosphate to enable comparatively light annual dressings to maintain the productivity premised by climatic conditions where moisture is not extremely limited. Lower dressings of phosphate have been optimal under reduced rainfall.

As the supply of available phosphate in the soil increases and organic enrichment proceeds either through dressings of phosphate alone or through the application of mixtures of phosphate and trace elements, two opposite sets of factors begin to operate, namely, depletion on the one hand, and improved availability through the medium of active plant roots, on the other. The balance between these must differ considerably under varying conditions of climate, soil, and management.

Copper and zinc are both applied as sulfate. Dressings range from 4 to 12 lbs. per acre. Molybdenum is applied as the trioxide at 1 oz. per acre. Only very small percentages of the amounts added are recovered in the herbage; residual effects are evident for 3 to 5 years. Dressings of copper or zinc sulfate exceeding 14 lbs. per acre may prove toxic. The return of copper, zinc, and molybdenum in the feces of grazing animals is considerable. All trace elements are applied to pastures in mixtures with superphosphate, prepared at the factory in accordance with technical recommendations.

Environmental Control

The South Australian environment has been classified into edapho-climatic units which differ widely in critical features. Of these, soil composition (see Fig. 1), the seasonal incidence of the rainfall in relation to light and temperature, the capacity of the soil to retain moisture through the profile under field conditions, and pH are among the most important.

An endeavor is made, in Table 1, to illustrate environmental properties that have closely governed herbage production where the moisture supply, in relation to soil retentivity, light duration, and temperature, is sufficient to meet the transpiration requirements of almost 2 tons per acre of dry pasturage. This quantity, if nutritionally suitable, can maintain 4 well-grown sheep to an acre, or a beast to 2 acres.

TABLE 1

Environmental Properties of Edapho-Climatic Units in South Australia

	pH	Mean wet period (mos.)	Climatic summation index	Dry herbage potential (tons/acid/ann.)	Legumes	Grasses	Deficiencies							Toxicities	
							N	P	Cu	Zn	Mo	K	Mn	Na Mg Cl SO_4	Organic derivatives
Calcareous sands	8.0	8	12	2.6	*M.*[1] *sativa* *M. truncatula*	*Phalaris*[3]	*	*	*	*	–	0		–	–
Rendzinas	8.0	8	14	3.1	*T.*[2] *fragiferum*	*Phalaris* *Lolium*	*	*	*	*	–	–		+	–
Peats	7.0	9	15	3.3	*T. repens* *T. fragiferum*	*Lolium*[4] *Dactylis*[5]	*	*	*	*	–	0	+	–	–
Irrigable clays	7.0	12	28	8.0	*M. sativa* *M. truncatula* *T. repens*	*Chloris*[6] *Lolium*	*	*	+	+	*	–	–	*	–
Red-brown earths	6.5	7	10	2.2	*T. subterraneum* *M. sativa*	*Phalaris* *Lolium*	+	*	–	–	–	–	–	–	–
Podsolized sands	6.0	8	13	2.8	*T. subterraneum*	*Lolium* *Phalaris*	*	*	–	0	+	–	–	–	–
Laterized podsols	6.0	8	12	2.7	*T. subterraneum*	*Phalaris* *tratium*	*	*	–	0	*	0	–	–	–
Siliceous sands	6.5	7	9	1.8	*T. subterraneum* *M. sativa*	*Phalaris* *Ehrharta*[7]	*	*	*	*	0	0	–	–	–
Heath podsols	5.5	9	14	3.0	*T. subterraneum*	*Phalaris* *Holcus*	*	*	+	+	+	*	–	–	*

[1] *M.* = *Medicago*
[2] *T.* = *Trifolium*
[3] *P. tuberosa*
[4] *L. perenne, L. subulatum*
[5] *D. glomerata*
[6] *C. gayana*
[7] *E. calycina*

* Acute
+ Secondary
0 Concerned

The extensive data and familiarity with practical conditions on which the classifications are based has been generalized to emphasize contrasts and illustrate in condensed form a method of integrated approach which can be utilized to determine future potentialities for agricultural development. The measures employed are indices subject to revision as further data from survey and experiment become available; but they illustrate the types of attribute to which measures of nutrients may be related.

The mean wet period is the resultant of monthly rainfall and evaporation from the soil. The season of influential rains varies considerably from year to year. The climatic index is derived from the product of the mean monthly length of day and the mean increase of temperature in °F above 45°F per month, summated to cover the period of the year over which moisture is available, and then divided by 80. This value is a generalized mean product of °F (above 45°F) light hours for a single month required to transpire an inch of water through the leaves of growing herbage in the Adelaide environment during the same month, based on investigations of water requirement and field production in relation to climatic factors over a period of 25 seasons.

Each unit of the summation index indicates the need for approximately 1 inch of water for transpiration and the production of 4-10 cwt. of dry herbage per acre, according to the relative rates of growth and transpiration. Herbage grown at Adelaide is characterized by an average transpiration ratio of about 500 (38); and experiments with tropical grasses at this center have indicated that their high rate of assimiliation reduces the ratio to approximately 200 at favorable temperatures and humidities. In the absence of studies of transpiration in tropical areas, estimates are speculative; but on the basis of the available data, mean air temperatures of 75-80°F throughout the year would indicate a summation index of 60, or a transpirational need of 60 inches, and a growth potential of 30 long tons of dry matter per acre. A critical minimum value of 3 p.p.m. of Cu in the dry matter would require 0.2 lb. of elemental copper from the soil in a single year, thus involving an average concentration of .015 p.p.m. of Cu in the water absorbed. This is

15 times the concentration (1γ per liter) found by Piper (29) as sufficient to supply plant needs, if maintained, in the Adelaide environment.

A minimum of 2 per cent nitrogen in the plant would require 1500 lbs. or $3\frac{1}{2}$ tons of sulfate of ammonia, and 0.5 per cent of P_2O_5, 375 lbs. P_2O_5, or nearly a ton of superphosphate. These figures indicate the key to the problem of animal production in the tropics. Much attention has been paid to improvement along genetic lines, but it is probable that the supply of nutrients from the soil rarely approaches that needed to provide requisite concentrations in the vast bulk of plant material induced by climate.

The nitrogen requirements of such herbage cannot be supplied economically through nitrogenous fertilizers; but if future work is competently orientated, legumes will be found which can assist at least in making good the deficiency of nitrogen, provided their needs for trace elements and phosphate are studied, and provided the soil deficiencies that no doubt exist are overcome, as they have been in some temperate regions. Albrecht (1) has emphasized the need to employ yield and amino acid composition of protein as major criteria of production per acre.

The rate of water loss compared with the rate of production will depend on the plant variety, stage of growth, evaporation rate as determined by atmospheric conditions, and the nutrient status within the plant. Water is transpired with greatest economy in relation to production under the conditions of light, temperature, humidity, and nutritional balance that are most favorable during the periods of active growth.

The total yield of a herbage plant association decreases with increased frequency of grazing or mowing, and transpiration decreases almost proportionately, so that loss of moisture through the leaves is reduced by pruning to a lesser volume. The reduction of yield or water transpired may be 50 to 70 per cent, yet the quantity of nitrogen gained from the soil can at the same time be trebled (38). The concentrations of nitrogen and phosphorus moving into the plant have been increased 650 to 800 per cent and 220 to 230 per cent respectively, as a result of frequent defoliation. The application to management, especially under exceptionally favorable conditions for growth, is obvious.

Profound changes are induced in a herbage sward by the introduction of new varieties, mineral treatment and grazing management. The adoption of such measures may greatly affect the concentrations of specific nutrients, both outside and within the plant. Practically all these modifications can now be brought within the control of man, provided the essential factors that have been touched on in this paper are competently investigated and integrated for the major types of contrasting environment concerned.

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Discussion

Dr. Arnon: It might be of interest to mention that recently Dr. Meagher of California has completed experiments which show that legumes require molybdenum, quite apart from the nitrogen fixation process. Legumes, even when supplied with nitrate, still require molybdenum as an essential element. This is in no conflict with the agricultural picture, as Dr. Trumble has pointed out, for under those conditions molybdenum may be particularly important for nitrogen fixation. I think these results would indicate that molybdenum is certainly required for more than just the symbiotic bacteria which are necessary for the nitrogen fixation of the legumes. In order to demonstrate the requirements for molybdenum over and above that required for nitrogen fixation, it is necessary to grow the plant in a molybdenum-deficient medium over a period of at least one generation in order to dilute out the amount of molybdenum initially present in the plant at the starting point.

Dr. Trumble: We have never doubted the importance of molybdenum in the nutrition of the host plant per se. However, even in those cases in the non-legumes or legumes without nodules which have been grown in molybdenum-deficient media, I have never seen an apparently molybdenum-deficient plant looking as greatly different from the control plant as in other deficiencies.

Dr. Arnon: Yes, I think that is true, but I think this is due to the fact that you have these two important unavoidable sources of contamination: this is, from your chemicals and from the biological sources, the reserve molybdenum carried by the seed. Therefore, I think it is necessary that one demonstrate a deficiency of this type by passing the plant through several generations in this deficient medium. I would like to make the following observation in regard to the essentiality of an element for plant growth. The important aspect in considering an element essential is whether or not the species can produce viable seeds; that is, can it perpetuate itself? If the plant is unable to reproduce itself over several generations in this deficient medium, then I think this is a criterion which can be used in the classification of the essentiality and non-essentiality of elements.

Dr. Steinberg: How would you consider asexual plants under this classification?

Dr. Arnon: In those circumstances one would be dealing with a clone, or in the case of *Aspergillus* with spores, which would be used as the initial inoculum. When the clone or spores grow in this deficient medium, they would form a plant which would have less reserve than previously. By using a part of this plant as an inoculum, you would finally get to a point where the plant would no longer reproduce itself if the element in which the medium is deficient is essential.

Dr. STEINBERG: The point I am trying to make is that the criterion is really one of growth, and that you cannot use reproduction alone as the criterion.

Dr. ARNON: That is true. I think that if we would substitute the word survival for growth or reproduction we would both agree that it wouldn't make much difference whether it was a sexual or an asexual plant.

Dr. TRUMBLE: It is simply a matter of exhaustion of the particular element by dilution, isn't that right

Dr. ARNON: Yes, that is essentially what it amounts to.

Dr. STEINBERG: How does molybdenum deficiency affect plants in the field with regard to growth and seed production?

Dr. TRUMBLE: There is a tendency to obtain stunted growth with very poor seed production.

Dr. TALBOT: What is the concentration of molybdenum in the plant itself?

Dr. TRUMBLE: One-tenth part per million would be about borderline for deficiency in subterranean clover; but the concentration may vary from 0.01 to above 50 p.p.m. and at this higher value the plant remains healthy.

Dr. TALBOT: It is rather interesting that the amounts you are talking of, namely an ounce per acre, are in an order of magnitude that we now know to be effective in one or two human situations. It is a ridiculously low amount.

Dr. TRUMBLE: That is the reason I would think the name " minor element " should be abandoned. With extremely small traces the effect is great.

Dr. VALLEE: What would be your suggestion of a substitute for the term " minor element "?

Dr. TRUMBLE: I like both " trace element " and " micronutrient," and think either a good one, but the objection has been made that an element occurring in trace amounts may not invariably function as a nutrient.

Dr. VALLEE: I am wondering whether the term " oligometal " would not be a good substitute? It has been used in the French literature.

Dr. ARNON: At an international conference at Rothamsted in 1947 this was one of the points of heated discussion. I would like to mention some of the arguments that were produced for and against some of the terms. The objection to the term " minor element " was that it implies to the student and to the non-technical person that minor elements are of lesser importance than the so-called major nutrients. This, of course, is not true. The objection to the term " trace element " is that at the present time we have analytical methods available which allow us to actually measure them, and they can no longer be considered, therefore, as merely occurring in trace amounts. So far as the micronutrient term is concerned, if we would all agree on certain criteria as to what determines a nutrient, then the objection which was just quoted is not perhaps valid. With regard to the term "microelements," chemists would object that no elements are microelements or macroelements. The term "oligometal" was brought up by the French and Dutch investigators at the conference. The term has historical respectability but has scientific obscurity. Since the term has never taken hold in the English literature, it is perhaps just as well not to give it currency now.

THE NUTRIENT ELEMENT CONTENT OF NATIVE FORAGES IN RELATION TO LAND FORMS AND SOIL TYPES IN THE NORTH CAROLINA COASTAL PLAIN

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INTRODUCTION

A deficiency of cobalt as one cause of nutritional troubles in cattle in eastern North Carolina probably was first recognized about 1940 by Dr. Branch Moore, a veterinarian in Kinston, North Carolina. According to Dr. Moore (personal communication), this nutritional deficiency had been observed for many years to occur in cattle in the early spring in Wayne, Lenoir, Green, Pitt, Craven, Jones, and Onslow counties. Affected animals had poor appetites that resulted in malnutrition, although feed was abundant (Figs. 1A and 1B). The trouble frequently terminated in the death of the animal. Dr. Moore could correct the trouble and obtain recovery of appetite within a week or less by administering small doses of an aqueous solution of a cobalt salt.

At about the same time Professor John Foster of the North Carolina State College of Agriculture was conducting grazing experiments in cooperation with the U. S. Bureau of Animal Industry and the U. S. Forest Service in the Hoffman Forest in Jones County (personal communication). Cattle transferred to this area from the Blackland Station at Wenona, North Carolina, rapidly developed an unthrifty condition, although the forage types available were the same in both areas. The first time a transfer was made, the animals became so unthrifty it was necessary to return them to Wenona to save them. Later, cobalt chloride was added to the mineral mixture supplied the animals in the Hoffman Forest area, and a significant improvement in their health resulted.

The recognition of cobalt as a limiting factor in animal growth and health in eastern North Carolina suggested the possibility of obtaining basic information concerning the relationship of soils and other natural features to these troubles. During 1942 and 1943 some preliminary studies were made (Beeson et al., 2) which confirmed the fact that the cobalt content (0.02-0.07 p.p.m.; average, 0.04 p.p.m.) of the forage at the Hoffman Forest area was not adequate for cattle (Beeson, 1). No systematic study of these relationships was begun, however, until the summer of 1946.

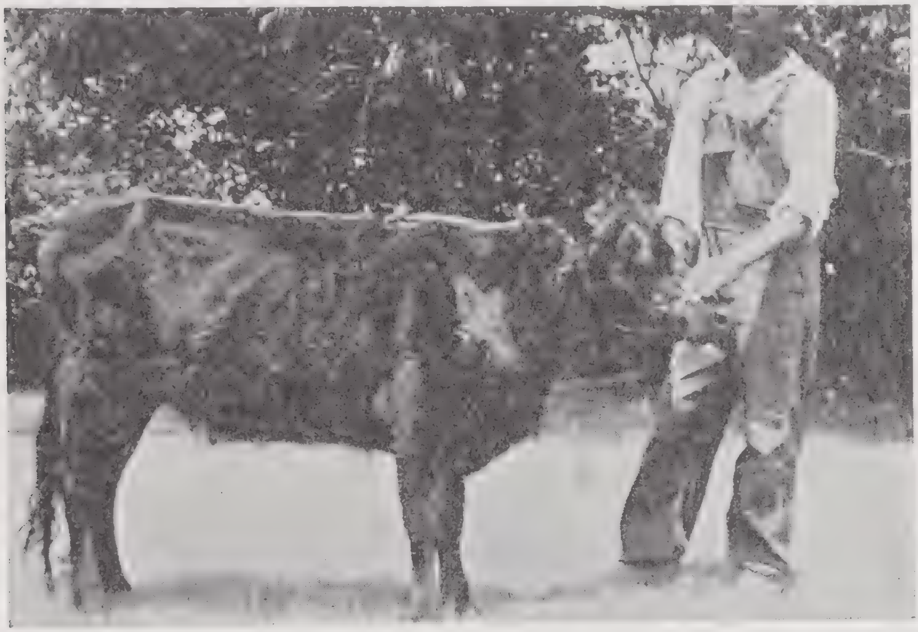

FIG. 1A. Two-year-old heifer suffering from a deficiency of cobalt in the forage in the Coastal Plain region. Note the general emaciation, particularly the roughness of the coat. The bone structure is good.

PRELIMINARY SURVEYS IN 1946 AND 1947

Samples of forage were collected in May, 1946, from a number of farms in Pendar, Onslow, Carteret, Jones, and Lenoir counties where cobalt deficiency had been diagnosed by Dr. Moore or Pro-

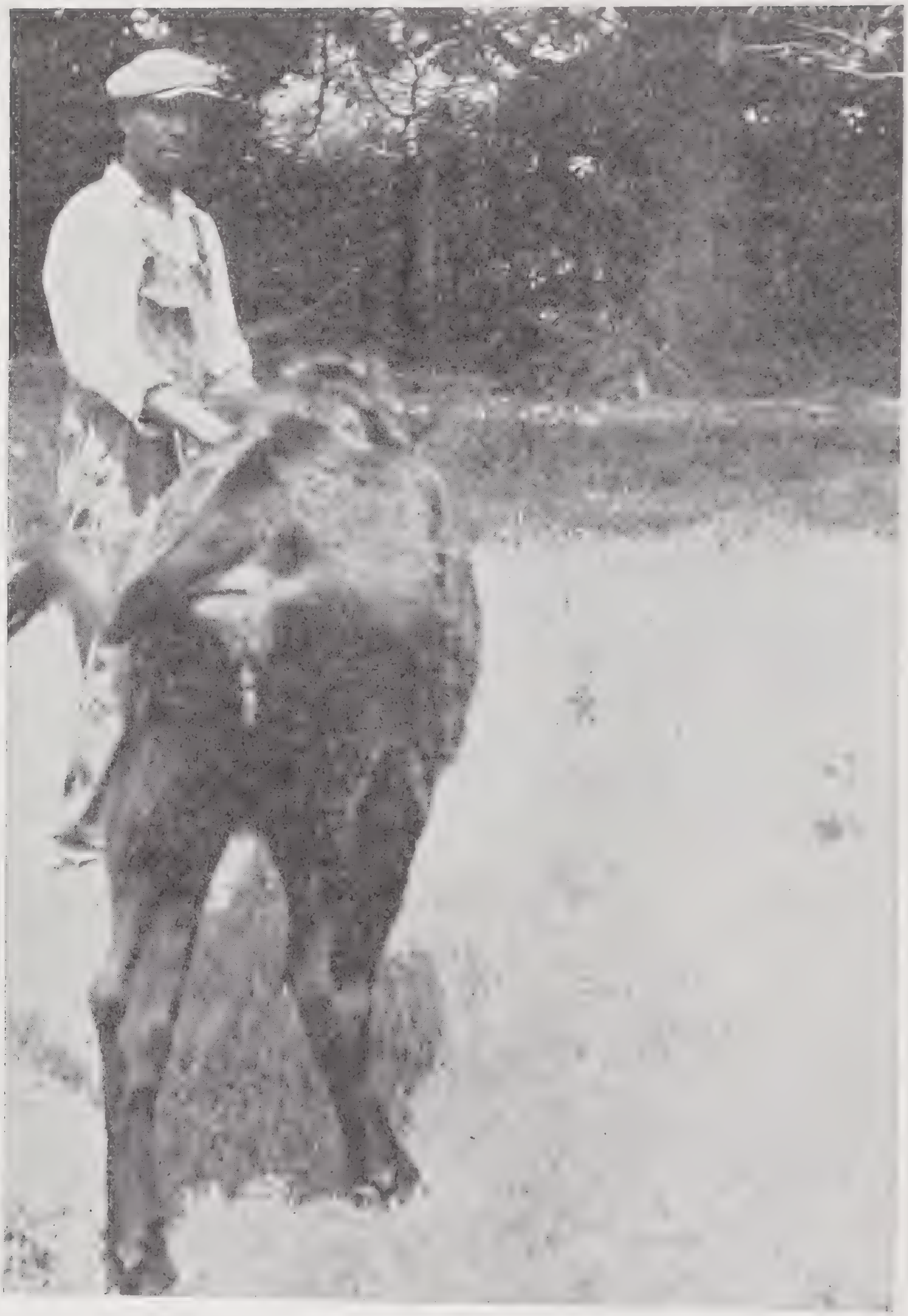

FIG. 1B. Another view of the heifer shown in Fig. 1A.

fessor Foster. Samples were also taken from farms apparently free of this trouble. The soils at each sampling location were classified by Professor W. D. Lee of the North Carolina State College. Biswell et al. (3) determined that the principal range plants eaten by cattle included the reed, *Arundinaria tecta,* and the bluestems (*Andropogon* spp.), particularly in the spring. Accordingly, for this study these plants were sampled in most instances, although some white clover and lespedeza samples were also taken. In the following year, May, 1947, it was decided to enlarge the area under study and not to restrict the forage sampling to farms selected on a basis of known occurrence or non-occurrence of troubles. The procedure was essentially a random sampling of a large area of several counties.

TABLE 1

Classification of Locations According to the Cobalt Content of Moisture-Free Forage Samples

Year	Total no. of samples	No. of locations	No. of soil types	Cobalt content of forage (p.p.m.)—per cent of total locations			
				0.01–0.03	0.04–0.07	0.08–0.11	0.12+
				%	%	%	%
1946	71	31	11	13	49	29	10
1947	110	55	10	30	53	13	2

A summary of the data obtained in the first two years of this study is presented in Table 1. If the range from 0.08 to 0.11 p.p.m. is accepted as being an adequate quantity of cobalt necessary for good nutrition in cattle (Beeson, 1), it is clear that less than half of the native forages from locations where samples were taken meet this requirement. A careful examination of the soil data and other pertinent facts did not reveal any consistent relationship of soil type to the cobalt content of the plant, although the occurrence of trouble in cattle seemed to be related in some manner to elevation.

To illustrate this relationship, a diagram showing the topographical features of Lenoir County, North Carolina, is presented in Fig. 2. Nutritional troubles in cattle were of frequent occurrence in the lowland areas adjacent to the river (A) and of less frequent occurrence on the upland of intermediate height. On the highest upland these troubles had never been observed (personal communication from Dr. Branch Moore). Further analysis of the data summarized in Table 1, however, did not reveal any statistically significant differences in forage composition with respect to these three areas. The variation in composition of the materials from any one area was great and prevented any attempt to determine a significant difference or trend from one area to another. It was apparent from these data that a more intensive as well as more extensive investigation of the composition of forages would be required if relationships of forage composition to soil conditions, land forms, or physiographic regions were to be established.

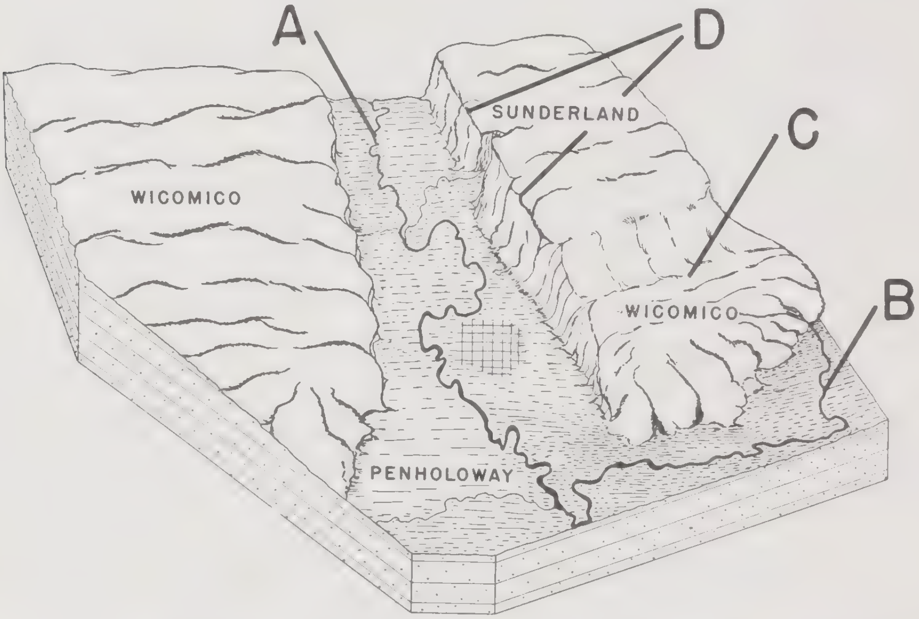

FIG. 2. Diagrammatic representation of typical Coastal Plain topography where a deficiency of cobalt in cattle is commonly found in the Penholoway and Wicomico marine terraces, but not on the Sunderland terrace. The elevation is greatly exaggerated.

Preliminary to any extensive sampling plan, a detailed study was made of the area depicted in Fig. 2. An examination of aerial photographs clearly showed that the main river valley (A, Fig. 2) in this instance was not normal, in that meander swings, particularly against the right bank (D), were not evident. In contrast to this, remnants of such swings were prominent at several levels in the sides of the valley cut by the tributary (B) in the lower right-hand corner of the diagram. Furthermore, on the upland labelled as Sunderland there is an abandoned but well-preserved stream bottom ending in a hanging valley as it approaches the scarp at the Sunderland boundary (D). It is apparent that this stream connected at one time with a body of water at a level very close to the Sunderland surface.

Johnson (10) and Cooke (5, 6) have described a series of terraces on the Atlantic Coastal Plain that are probably of marine origin. According to Cooke, these geographic divisions of the Coastal Plain stand less than 270 feet above sea level and comprise, in South Carolina, more than two-thirds of the total Coastal Plain area in that state. The landward limit of each terrace is defined by the level shore line at which the sea and its estuaries stood when the terrace was under water. The seaward limit is the shore line of the next lower terrace.

In its discussion of the South Carolina Coastal Plain, Cooke (7) stated that at the end of the Pliocene epoch there was a drowning of the coastal region as far inland as the landward margin of the present coastal terraces. The sea then probably stood about 270 feet higher than its present level. Alternate high and low levels of the sea have occurred since that time, with flooding and deposition of clay, sand, and gravel. At each temporary stand of the sea the waves cut back into the headlands, and these wave-cut and wave-built features remained to mark the abandoned shore line.

This process could account for the particular topographic features diagrammed in Fig. 2. The extraordinarily wide and flat " river valley " (A) with its steep and straight sides is probably a portion of a marine terrace, and since the maximum elevation of this area is about 60 feet, it would be classified as the Penholoway terrace. Further investigation of the elevations in this area showed two additional and distinct regions, one at a maximum of 100 feet, which is designated by Cooke as the Wicomico terrace, and the third with a maximum elevation of 170 feet, classified as the Sunderland terrace.

Further evidence supporting the hypothesis of different marine formations in this region was found in a series of low-lying dome-shaped hillocks at location C in Fig. 2. This is on the well-defined landward border of the Wicomico terrace. The hills could be remnants of ancient sand dunes developed on the shore when the sea was at this level.

Experimental Plan in 1948

Postulating the existence of discrete land formations in the area under study suggested the possibility of variations of importance in the nature of the parent materials associated with them. It was reasoned that this would modify plant composition, which in turn could account for the occurrence or non-occurrence of nutritional troubles in cattle. It was evident that the area previously studied was too small to permit an adequate determination of necessary basic facts. Accordingly, it was decided to study the entire Coastal Plain area in North Carolina, the objective being to discover, if possible, through this more comprehensive approach, those soil and geological factors associated with the occurrence of nutritional troubles in animals.

Topographic maps are available for less than half of the quadrangles in the North Carolina Coastal Plain. Where available, however, these maps were used to obtain the outlines of each marine terrace. Other available data and actual investigation in the field with the help of relatively crude instruments (a barometer and a hand level) served to establish the boundaries in other areas. As a result, a map (Fig. 3) was constructed, showing the general outlines of the boundaries of the seven marine terraces. The symbol used for each terrace, the name of the terrace, and the maximum elevation on the landward side are given in the legend of the figure. The boundaries are irregular, but in a general way parallel the present coast line. At several points, a terrace disappears, and as a result there is a steep escarpment at the junction of the two terraces that normally occur on either side of the missing one. This is illustrated in Fig. 3 where the Penholoway and Sunderland terraces join because of the absence of the Wicomico terrace.

With the establishment of a consistent and progressive variation in land forms throughout this region, it was possible to outline a systematic procedure for sampling vegetation for chemical analysis. The first criterion for the selection of samples was, of course, elevation as represented by the seven terraces. In addition, the Piedmont Plateau adjacent to the Coastal Plain was included. The second criterion was strictly a characteristic associated with soil type, namely, the amount of internal drainage. Two extremes, the excessively drained soil, represented by the Norfolk fine sands, and the poorly drained soil, represented by the Portsmouth fine sands, were selected.

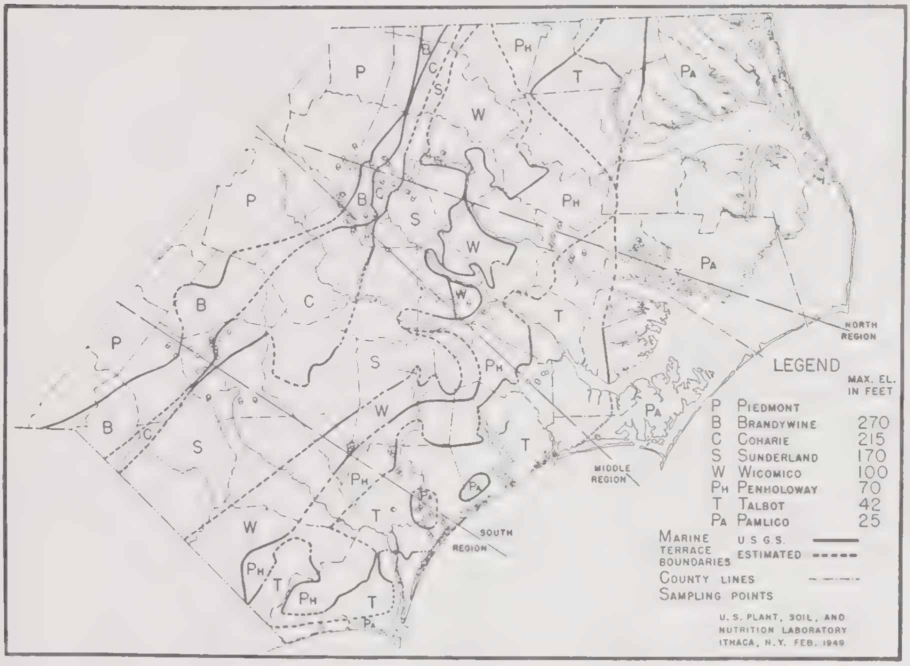
NORTH REGION
MIDDLE REGION
SOUTH REGION
LEGEND
MAX. EL. IN FEET
P PIEDMONT
B BRANDYWINE 270
C COHARIE 215
S SUNDERLAND 170
W WICOMICO 100
PH PENHOLOWAY 70
T TALBOT 42
PA PAMLICO 25
MARINE TERRACE BOUNDARIES U.S.G.S.
ESTIMATED
COUNTY LINES
SAMPLING POINTS
U. S. PLANT, SOIL, AND NUTRITION LABORATORY ITHACA, N.Y. FEB. 1949

FIG. 3. Map of the Coastal Plain in North Carolina showing the boundaries of seven prominent marine terraces and the sampling locations used in this study.

As a third criterion, the geographical location on the Coastal Plain was considered. The whole Coastal Plain was divided into three regions designated as north, middle, and south. The boundaries of these three regions were determined roughly by the drainage systems of the principal rivers in the Coastal Plain. These rivers are all consequent to the Coastal Plain and were responsible for transmitting much of the material of which the terraces in each region are composed. The transects of the map (Fig. 3), labeled north, middle, or south region, respectively, indicate the approximate middle of each region.

Having established the three basic criteria for sampling, there remained the problem of actually selecting the sampling sites. Near the center of each region (north, middle, or south) and on each terrace, three locations were chosen at random. At each location samples of vegetation were taken in duplicate from a well-drained and from a poorly-drained soil in close proximity. Hence the latter two plots were considered subplots, since only one was chosen at random. Thus the total number of samples taken under these conditions was 288.

It was recognized that the same species would not always be available under both drainage conditions. The reed or cane (*Arundinaria tecta*) is most commonly found in poorly-drained places; in fact, it is a good indicator of an amply supply of moisture. Broomsedge (*Andropogon virginicus*) is found both in wet and dry areas, but it can always be found in well-drained grazing areas. These two species were selected for sampling, therefore, as being most representative not only as forage but also because of their widespread occurrence. On each elevation and in each region an additional sample of broomsedge was collected from exactly the same plot as the reed. In this way the compositional relationship of the two species was determined.

In general, the broomsedge was immature but was optimum as a forage for grazing. The reed leaves were well developed. Only leaves of good color were selected, since the physiological age of the leaf on any stem can vary. Thus necrotic leaves or very young, immature leaves were avoided. Only the leaf is palatable to cattle. The entire sampling was completed within three weeks. Although some differences in maturity of the broomsedge probably occurred in this time, it is doubtful if it was of great importance.

The locations for sampling were selected entirely at random within the region and the marine plain. The choice of a plot was limited—

seriously in some instances—by a preponderance of cultivated areas, particularly where the drainage was good. Two criteria for selection of a site were established. First, plots where natural conditions prevailed as nearly as possible were sought. This limited the selection to forest land or to land abandoned for a considerable time. Much of the forest land suitable for grazing in this area is also poorly drained. Most of the better-drained land is under cultivation, particularly in the lower Coastal Plain. Secondly, any area chosen was sufficiently remote from roads and other sources of dust to minimize contamination of the sample. This criterion was the most difficult to satisfy, since rather large areas were involved. Fortunately, the 1948 spring season had been a wet one, and contamination by dust from roads and fields was not considered to be important.

Collection and Preparation of the Sample

Broomsedge was clipped with grass shears, and the new growth was sorted out from all other vegetation. Reed leaves were plucked by hand from the entire stalk. In both cases samples were taken from plants over a fairly wide area until at least 100 grams of material was obtained. The duplicate sample was taken nearby but not over the same area.

The samples were oven-dried and cut into small pieces with steel scissors. This method avoided the contamination often experienced with the usual mills (Hood et al., 9). A solution of ten grams of dry plant tissue was prepared according to the method of Ellis and Thompson (8). The following analytical procedures were used: for the cobalt, the nitrosocresol method (Ellis and Thompson, 8); for copper, the sodium diethyldithiocarbamate method; for manganese, the periodate method; for iron, ortho-phenanthroline method; and for phosphorus, the colorimetric method (Parks et al., 17).

Experimental Results

All analyses were made in duplicate on each of the 288 samples of vegetation. The variation attributed to each factor was computed by an analysis of variance (Table 2). In the statistical analysis of the data the experimental plan was treated as a modified split-plot design. Each one of the three locations within each region—north, middle, and south on each terrace—was considered to be a whole plot. Each whole plot contained a well-drained and poorly-drained subplot. The variation among the three whole plots within each region (2 degrees of freedom), pooled over all the three regions in each terrace, constitutes Error A in Table 2. It is used for testing elevations, regions, and their interactions. Error B is a measure of the random variation within the subplots. It is appropriate for testing the significance of drainage and its interactions with region and elevations. It is evident from Table 2 that statistically significant differences in the composition of the plant were associated with these factors, although the effects on all elements are not uniform. Thus, only drainage was significant in relation to cobalt content, while all factors affected iron content significantly, and many interrelationships were also involved. A detailed compilation of the

TABLE 2

General Analyses of Variance for All Factors Considered Except Species

Source	Degrees of Freedom	Cobalt	Copper	Iron	Manganese	Phosphorus
		Mean Squares				
Elevations (E)	7	.004333	22.97*	60,720†	366,617*	.02489*
Linear	1	....	23.12	389,957†	2,138,605†	.03133
Quadratic	1	....	56.93*	23,281	22,283	.05844*
Regions (R)	2	.001965	9.95	27,567*	459,647*	.04410*
ER	14	.009383	20.47*	12,537*	270,841*	.01457
Error A	48	.005270	8.53	6,396	125,705	.00963
Drainage (D)	1	.075854†	443.77†	76,897†	40,210	.00307
ED	7	.000662	13.37	10,050†	83,148	.00832
RD	2	.004230	.56	48,037†	101,502	.02525
ERD	14	.004305	6.53	18,668†	123,166*	.00453
Error B	48	.005232	6.42	2,755	62,456	.00806
Sampling error	144	.002215	5.00	1,617	30,690	.00132

* Significant, $P \leq 0.05$.
† Highly significant, $P \leq 0.01$.

data is not presented here, but they are summarized in tables and graphs in such a way as to emphasize their relationships to the factors studied. For this purpose, each element will first be considered alone.

Cobalt

The cobalt content of vegetation was significantly affected (99: 1) by the moisture relationships of the soil. Thus the average on the well-drained soils was 0.05 p.p.m. of cobalt in the dry matter, while the average for the poorly-drained soil was 0.03 p.p.m. Since the species of plant selected was not the same on both types of soil, a comparison was made of the broomsedge and reeds where both were growing on the same soil. The average cobalt content of the broomsedge samples was 0.07 p.p.m., while that of the reeds was 0.06 p.p.m. This is not a statistically significant difference (Table 3). It can be concluded, therefore, that the effect of drainage on cobalt content was direct and difference of species was not a factor.

TABLE 3

ANALYSIS OF VARIANCE IN DATA OBTAINED FROM CHEMICAL ANALYSES OF THE TWO SPECIES, *Arundinaria tecta* AND *Andropogon virginicus*, GROWING IN THE SAME LOCATIONS

Source	Degrees of Freedom	Cobalt	Copper	Iron	Manganese	Phosphorus
		Mean Squares				
Species (S)	1	.0011	18.54†	5241*	704	.0009
Regions (R)	2	.0040	.71	5788†	8434	.0040
Elevations (E)	7	.0036	1.46	2495*	47842*	.0033
Error	35	.0015	1.29	791	17477	.0015

* Significant, $P \leq 0.05$.
† Highly significant, $P \leq 0.01$.

The general analysis of variance does not reveal any other factor than drainage significantly affecting the cobalt content of either the reeds or the broomsedge. Nevertheless, it is instructive to classify the data on the basis of cobalt content and in relation to the marine terraces. Tables 4 and 5 relate these data to the requirements of the animal for cobalt. Thus, 0.01 to 0.03 p.p.m. of cobalt in the forage is definitely deficient for both sheep and cattle. A level of 0.04 to 0.07 is deficient for sheep but borderline for cattle. A value greater than 0.10 p.p.m. of cobalt would probably supply a sufficient quantity for either animal species.

TABLE 4

Cobalt Content of *Andropogon virginicus* (Broomsedge) Sampled on Well-Drained Soils in the Coastal Plain of North Carolina, May, 1948

Terrace	Maximum elevation	Average cobalt in dry matter	Distribution of samples according to their cobalt content (p.p.m.)			
			0.01–0.03	0.04–0.07	0.08–0.10	0.11+
	(feet)	(p.p.m.)				
Pamlico	25	0.05	12	4	0	2
Talbot	42	.05	9	7	1	1
Penholoway	70	.06	11	4	1	2
Wicomico	100	.04	7	10	1	0
Sunderland	170	.05	9	7	1	1
Coharie	215	.04	11	6	1	0
Brandywine	270	.06	5	10	1	2
Piedmont	...	.08	5	5	4	4

TABLE 5

Cobalt Content of Leaves of *Arundinaria tecta* (Reeds or Cane) Sampled on Poorly-Drained Soils in the Coastal Plain of North Carolina, May, 1948

Terrace	Maximum elevation	Average cobalt in dry matter	Distribution of samples according to their cobalt content (p.p.m.)			
			0.01–0.03	0.04–0.07	0.08–0.10	0.11+
	(feet)	(p.p.m.)				
Pamlico	25	0.03	12	5	1	0
Talbot	42	.04	8	8	2	0
Penholoway	70	.03	12	6	0	0
Wicomico	100	.03	12	6	0	0
Sunderland	170	.04	8	10	0	0
Coharie	215	.02	13	5	0	0
Brandywine	270	.03	8	10	0	0
Piedmont	...	.03	14	3	1	0

When the data from the well-drained soils are classified in this manner, it is apparent (Table 4) that a larger number of samples containing the high quantities of cobalt was found on the Piedmont Plateau than on any of the marine terraces. Thus, two-thirds of the samples obtained on the Pamlico terrace contained less than the minimum quantity of cobalt for cattle, whereas less than one-third of the samples on the Piedmont Plateau were in the extremely deficient class. Between the Pamlico and Coharie there was little difference in distribution of the samples, but some improvement is indicated on the Brandywine terrace. In contrast, the distribution of samples according to the cobalt content of vegetation on the poorly-drained soils is very uniform throughout the whole Coastal Plain and including the Piedmont Plateau. Only four samples out of the 144 contained more than the minimum required for cattle, and 60 per cent were deficient. The data from the well-drained soils when analyzed separately had a much higher error variance than did those from the poorly-drained soils. Consequently, when only the latter data were analyzed there were significant (19: 1) regional and region × elevation effects. The cobalt was lowest in the north and highest in the south region. Moreover, the nature of the effect in the south region differed with respect to the other regions in that the cobalt level on the two lowest terraces was higher than on the older terraces, whereas in the middle and north regions the highest values for cobalt were found on the higher terraces. However, these trends are of little practical importance because of the very low level of cobalt on poorly-drained soils throughout the Coastal Plain.

Copper

The overall analysis of variance (Table 2) shows that the copper content of vegetation was significantly influenced by elevation and that the effect was quadratic rather than linear. No significant variation in copper content could be associated with region (north, middle, and south). The effect of drainage was highly significant (99: 1), but an examination of samples of both broomsedge and reeds growing on the same soil indicated a definite species difference with respect to the absorption of copper (Table 3). Thus, the average copper content of the reeds was 6.8 p.p.m. of the dry matter while that of the broomsedge was 5.6 p.p.m. The overall average of reeds from the poorly-drained soils was 6.9 p.p.m. of copper in the dry matter and that of broomsedge from well-drained soils was 5.7 p.p.m. (Table 6). The agreement between these data and those obtained from the two species growing on the same soil indicate that the differences in copper content between reeds grown on poorly-drained soils and broomsedge grown on well-drained soils was probably a species difference rather than a difference associated with the moisture relationships of the two types of soil.

TABLE 6

Copper Content of Vegetation Sampled on the North Carolina Coastal Plain, May, 1948

Species	Moisture relationship in soil	Average copper in dry matter	Distribution of samples according to their copper content (p.p.m.)		
			3.1–5.0	5.1–7.0	7.1+
		(p.p.m.)			
Broomsedge	Well-drained	5.7	42	86	16
Reed	Poorly-drained	6.9	12	67	57

Since species and drainage effects are confounded, it was desirable to test the factors under study within species. No significant effects of elevation or region on the copper content of broomsedge growing on well-drained soils were found. There is, however, a significant (19: 1) linear effect in the data from the well-drained soils. This is interpreted as indicating a slight increase (less than 0.5 p.p.m.) in copper content with increase in elevation or age of the marine terraces.

There is a highly significant effect (99: 1) of elevation on the copper content of reeds growing on the poorly-drained soils (Fig. 4), and the nature of the effect varies somewhat (19: 1) in different regions. The magnitude and character of the effect of elevations or marine terraces on copper content are presented in Fig. 4. The parabolic trend in the data from the poorly-drained soils is highly significant (99: 1) and indicates a definite maximum on the Sunderland terrace. There is a regular increase in copper content of reeds up to that level, followed by an equally regular decrease until the highest terrace is reached. An average difference in copper content of about 1.5 p.p.m., or 20 per cent of the total, is involved.

Since an elevation × region interaction was indicated in the analysis of variance of the data from poorly-drained soils, a detailed study was made of the trend of copper content of reeds from different regions with respect to elevation. In each region a trend (Fig. 5) similar in character to that in Fig. 4 was obtained, with the maximum point occurring on the Sunderland terrace. In all except the middle region this trend was significant.

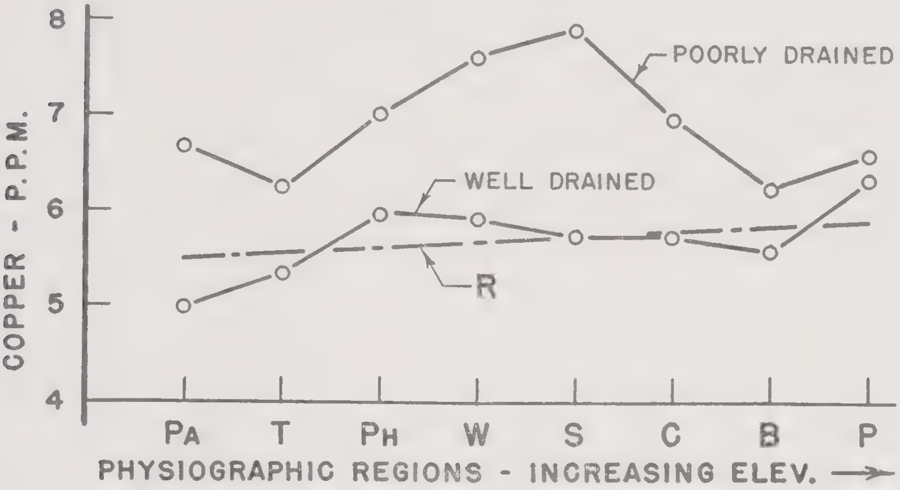

FIG. 4. Relation of the copper content of reeds (native vegetation) to soil drainage and to physiographic regions. Each point represents the average of 18 samples selected in duplicate from three regions (north, middle, and south) and three plots within each region. The trend is represented by the broken line R.

Other characteristics of these data can be obtained from a consideration of the distribution of samples according to their copper content, as shown in Table 6. Nearly three times more broomsedge than reed samples fall in the lowest copper class. The relationship is reversed in the highest copper range, again emphasizing the superiority of the reed as a carrier of this element.

Iron

The iron content of vegetation was significantly affected by a number of factors (Table 2). In particular, there is a highly significant linear effect in relation to elevations (or terraces). An examination of the data reveals that this is due to an overall increase in iron content from the lowest to the highest marine terrace. The analysis of variance indicates, however, several interactions in the data. Hence, the data were examined in detail with respect to all of these factors.

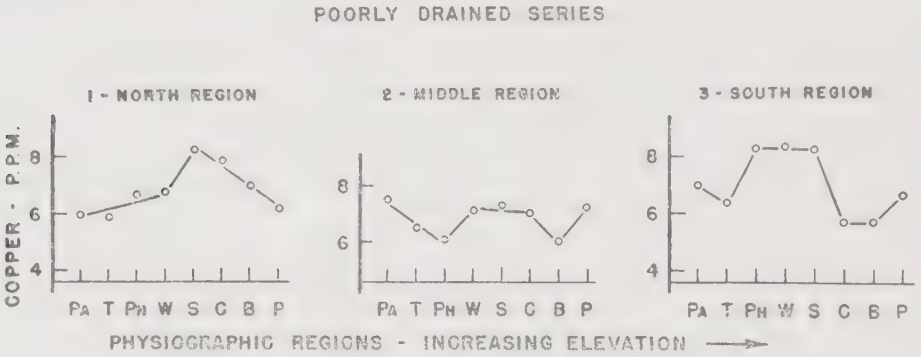

FIG. 5. Relation of the copper content of *Arundinaria tecta* to geographical location and to physiographic regions. Each point is the average of determinations on 6 samples selected in duplicate from three plots.

According to the analysis of variance presented in Table 3, the iron contents of broomsedge and of reed leaves vary significantly (19: 1) from each other when they are grown in the same area. The average values of 23 samples of each are 86 and 65 p.p.m., respectively, in the broomsedge and in the reed leaves. According to the general analysis of variance (Table 2), drainage, which of course is confounded completely with species, affected the iron content of the vegetation. The average iron value for broomsedge grown on the well-drained soils is 67 p.p.m, while that for reed leaves grown on the poorly-drained soil is 73 p.p.m. The difference is highly significant (99: 1). This is the reverse of the species study, and it is interpreted to mean that drainage conditions had an effect on iron content opposite to and greater than the species effect.

Since both regions and elevations as well as drainage affected iron content, the data were separated and plotted with respect to all of these factors (Figs. 6 and 7). Analyses of variance were also calculated separately on data from well-drained and poorly-drained soils, with tests for linearity in the data in relation to elevations. There are highly significant main effects and interactions in the data due to elevation and regions in both the well-drained and poorly-drained series. Where these effects are operating they are linear, with the exception of samples from the well-drained soils of the south region. Thus, from the Pamlico to the Brandywine terrace in the south (well-drained series, Fig. 6) there is no significant change in iron content, but on the Piedmont in this region there is a striking increase. No change in iron content occurs in relation to the terraces

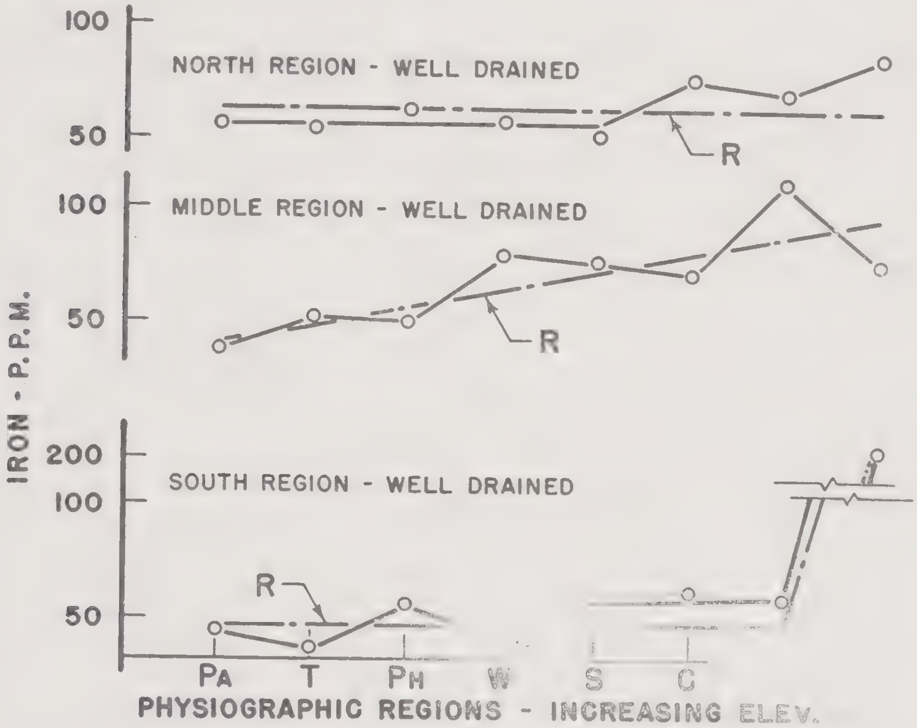

FIG. 6. Relation of the iron content of immature *Andropogon virginicus* to geographical location and to physiographic regions. Each point is the average of 6 samples selected in duplicate from three plots within each region. The trends are represented by the broken lines R.

in the north, but in the middle region there is a highly significant (99: 1) increase in iron with increase in elevation or age of terrace, as indicated by the broken line R (Fig. 6). The changes in both series are of some magnitude, being nearly three-fold in each case.

There is in these data a highly significant (99: 1) linear effect from south to north in the poorly-drained series. This is also evident from an inspection of the curves in Fig. 7. It is apparent, however, that the importance of the effect is at the higher elevations, since the iron content of the reed leaves is uniformly low in the lower and younger terraces.

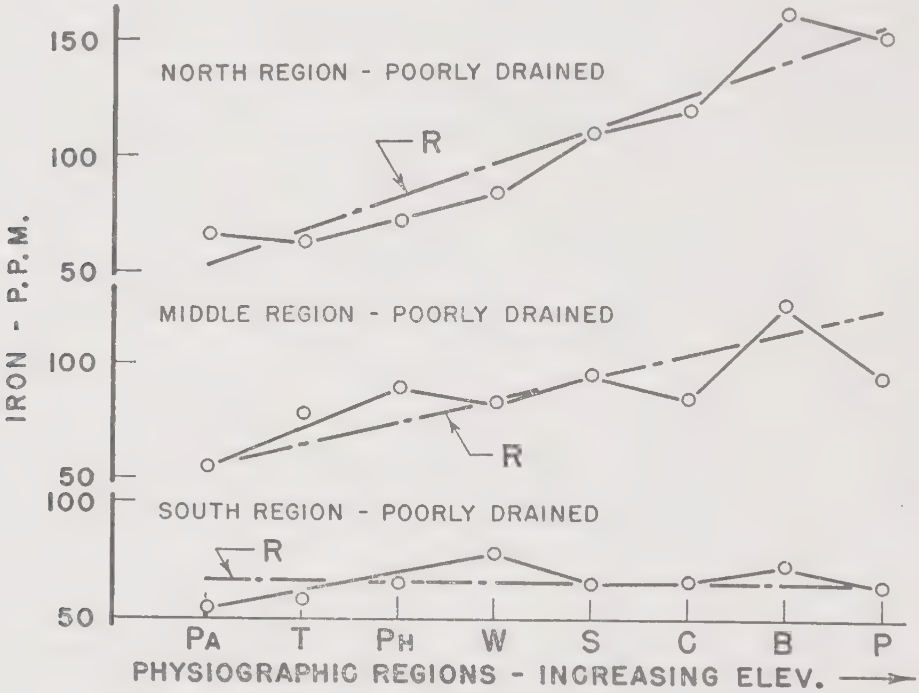

FIG. 7. Relation of the iron content of leaves of *Arundinaria tecta* to geographical location and to physiographic regions. Each point is the average of 6 samples selected in duplicate from three plots within each region. The trends are represented by the broken lines R.

Manganese

The manganese content of vegetation was significantly affected by elevation (terraces) and regions (north, middle, and south) in this study (Table 2). Drainage was not a factor, and a study of leaves of both species, broomsedge and reed, growing in the same location did not reveal any significant difference in their manganese content. Since neither species nor drainage was a factor it was possible to examine as a unit data from both well-drained and poorly-drained plots.

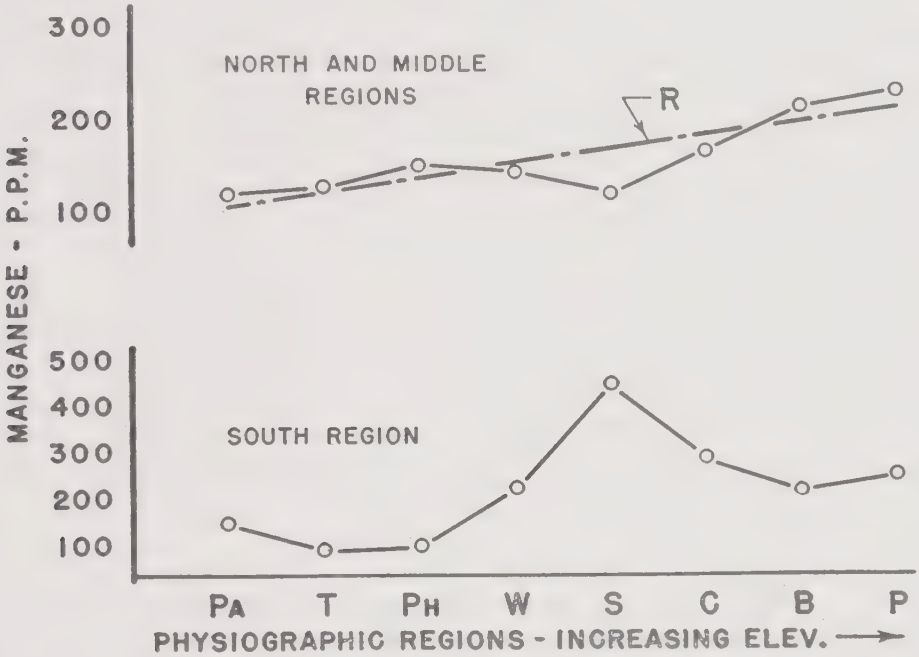

FIG. 8. Relation of the manganese content of native vegetation to geographical location and physiographic regions. In the top curve (north and middle regions) each point is the average of determinations on 24 samples selected in duplicate from two regions, three plots within each region, and two soils within each plot. Each point on the south region curve is the average of determinations on 12 samples selected in duplicate from three plots and two soils within each plot. The trend is represented by the broken line R.

A detailed analysis showed that the south region is different (99: 1) from the middle and north region. The data from the middle and north regions do not indicate any differences between these regions since the average manganese contents in the vegetation are the same for both. The combined data from the north and middle regions indicate a highly significant (99: 1) linear relationship (R, Fig. 8), one in which the manganese content of the vegetation on the highest and oldest soils is about double that on the youngest, low-lying soils.

A detailed study was also made of the data from the south region, which were significantly different from those of the other two regions. The combined data from both drainage conditions have been plotted in Fig. 8. There is a highly significant (99: 1) linear relationship in the data, indicating an increase in manganese content in the vegetation with increase in the elevation. There is also a significant (19: 1) parabolic trend, as is apparent in the curve. A further analysis of the data from the first five terraces (Pamlico through Sunderland) demonstrates significant linear and quadratic effects, indicating that the trends in Fig. 8 are real and not accidental. The minimum values occur on the Talbot and Penholoway terraces, and there is an overall increase to the Sunderland. Finally, an examination of the last four terraces (Sunderland through Piedmont) showed a highly significant (99: 1) linear effect, a decrease in the manganese content as the elevation increases, thus indicating that the maximum value on the Sunderland terrace is also significant.

Phosphorus

The phosphorus content of neither of the species sampled was affected by drainage, but significant effects (19: 1) of elevations and region are inherent in the data (Table 2). Species were not different in their phosphorus content (Table 3), and the regional effects were so small that they were of no practical nutritional importance. The best concept of the relation between phosphorus content and location (or physiographic region) can be had from an examination of the data from all regions (north, middle, and south) and from both drainage conditions. These data have been plotted with respect to elevation in Fig. 9. The analysis of variance (Table 2) shows a significant (19: 1) quadratic effect but no linear effect. The main quadratic effect apepars to be a maximum phosphorus content on the Penholoway terrace and a decrease from that elevation to the Piedmont (R, Fig. 9). The data from the Pamlico and Talbot terraces were tested against those from the Penholoway and found to be significantly different (99: 1).

The generally low level of phosphorus in the native forages on the Coastal Plain is illustrated by the fact that 17 per cent of all data fell in the range of 0.05 to 0.10 per cent of phosphorus in the dry matter. Fifty-five per cent of all determinations were in the range 0.11 to 0.16 per cent of phosphorus. Only four samples in 288 contained more than 0.22 per cent of phosphorus.

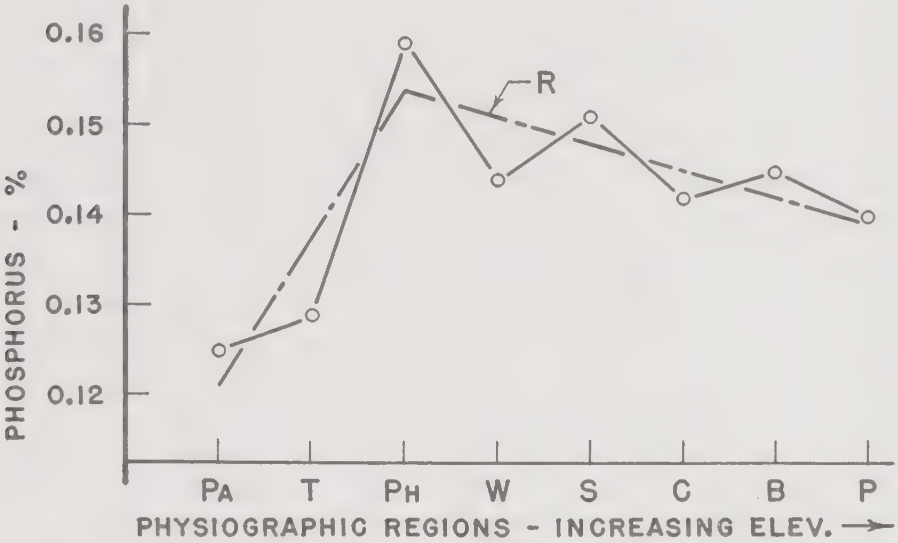

FIG. 9. Relation of the phosphorus content of native vegetation to physiographic regions. Each point on the curve is the average of 36 samples selected in duplicate from three geographical regions (north, middle, and south), three plots within each region, and two soils within each plot.

DISCUSSION

Although this study clearly demonstrates variation in the composition of native forage species in relation to physiographic regions, there is little evidence that permits assigning causes to these variations. Three types of variation are evident in the data: (1) an increase in the content of mineral as the age and elevation of the Coastal Plain terraces increases, (2) a maximum level of one or more mineral elements at some intermediate terrace, and (3) an effect of drainage or species, or both.

A discussion of manganese is helpful, however, in suggesting possible reasons for the distribution of other elements, such as copper, that show a maximum at an intermediate level. When the sea bordered the Coharie terrace and covered the Sunderland, the main drainage in the south region of the Coastal Plain was probably derived from a large area extending from Davie and Randolph counties into Caswell and Person counties to the northeast. The main stream carrying this drainage was probably what is now an extended consequent, the Cape Fear River. This river drains an area between the Roanoke on the north and the Pee Dee River on the south. An important source of materials carried down by the Cape Fear and its tributaries and deposited on the Continental shelf must have been the dark-colored quartz-free rocks, usually diabase, basalt, or metamorphic rocks of similar mineralogical composition. These rocks are the parent material of the Iredell and Davidson soils (Jurney et al., 11, 13, 15) [1]; and the manganese content of the parent materials of these soils ranges from 0.18 to 0.47 per cent of the oxide (Marbut, 16). In contrast, the predominating soil to the north and south, the Cecil, is derived from schists and gneisses of granitic origin which contain from 0.02 to 0.17 per cent of the oxide of manganese (Marbut, 16). According to Clarke (4), basalts may contain from 0.10 to 0.26 per cent of manganous oxide, while granites seldom contain more than 0.09 per cent and very often only a trace.

The importance of these facts in accounting for the unusually high manganese content of vegetation growing on the southern portion of the Sunderland terrace is clear. Unfortunately, no analogy can be found for the high copper content of the same terrace, since so few copper data are available for rocks and soils. Furthermore, the high copper content of the Sunderland terrace was characteristic for the whole terrace from north to south, and not for one segment. This would tend to cast some doubt on the proposition that the original materials transported to this terrace were richer in copper than those transported to other terraces.

It is more probable that the varying amounts of copper available to plants on these terraces is a function of age, weathering, and leaching. Cooke (6) suggesting that the exposture of the Sunderland terrace and the lower terraces was separated by the Illinoian glacial stage. This would make the Sunderland several hundred thousand years older than the lower terraces, and the time interval between it and the next younger terrace would probably be much greater than the interval between any of the younger terraces.

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[1] Suggested by Dean H. P. Cooper.

However, a consideration of all the data does not suggest an important interruption in trends up to and including the Sunderland. Thus, there are linear increases in the copper, manganese, and iron contents of vegetation, with only unimportant deviations on the Pamlico terrace. Phosphorus decreases from the Penholoway to the Piedmont without a significant deviation at the Sunderland or elsewhere. These observations obviously do not eliminate age as a factor, since weathering, leaching, and the number of vegetative cycles must have played important roles in mobilizing and dissipating the mineral elements in these soils. These effects are, in fact, counteracting each other in a manner that always permits one or the other to be dominant. In those cases where an element like iron increases regularly from the younger to the older terraces, factors favoring accumulation may be dominant. This situation may be equally true for manganese, except for the unusual situation on the Sunderland in the southern portion of the Coastal Plain.

It would be hazardous to speculate concerning the trends for phosphorus, since complicated chemical reactions are also concerned. Probably the most important fact concerning phosphorus is the relatively low value on the two youngest terraces. No explanation is available for this or for the maximum on the Penholoway Terrace.

Any consideration of the factors involved in the absorption of these elements by plants must take into account drainage conditions in the soil and at least the major factors associated with them. Highly significant differences in the cobalt, copper, and iron content of vegetation depending upon soil drainage conditions or species are inherent in the data. The cobalt content of *Arundinaria tecta* (grown on poorly-drained soils) is lower than *Andropogon virginicus* (grown on well-drained soils), and thus the cobalt content of the forage is low to the extent that cattle must depend on reeds from poorly-drained soils. Apparently the net effect of drainage and species is to provide a forage of somewhat higher iron content on poorly-drained soils, and species differences provide a higher copper content on the same soils.

Out of this confusion of relationships one thing stands out clearly in relation to trouble in cattle. If the cobalt content of *Arundinaria tecta* is low on poorly-drained soils, it is clear that the occurrence of cobalt deficiency in cattle may be more frequent in those areas having a high percentage of poorly-drained soils. Furthermore, it is the poorly-drained areas less adaptable to other kinds of management that are used for grazing. Thus more animals are available as indicators of trouble in areas with important acreages of such soil.

The distribution of well-drained and poorly-drained soils in two counties in North Carolina is given in Table 7. It is clear that the higher terrace (Sunderland) is associated with a much smaller percentage of poorly-drained soils in its total area. Hence, it can be assumed that a larger percentage of soils on this terrace is supporting native vegetation higher in cobalt. Likewise, in the younger terraces the proportion of poorly-drained soils and therefore the quantity of low-cobalt native forage is high. It should also be recognized that the size of individual areas of poorly-drained soils tends to increase as the elevation of these terraces decreases. Some such areas comprising thousands of acres are commonly used for grazing near the coast.

TABLE 7

Distribution of Soils According to Drainage in Two Counties of the North Carolina Coastal Plain (Jurney, et al., 1921, 1925)

County	Predominant Terraces	Well-drained soils	Poorly-drained soils
		%	%
Onslow	Talbot and Penholoway	39	57
Wilson	Sunderland	79	21

It cannot be concluded upon the basis of present evidence that cobalt is the only limiting factor in the nutrition of cattle on the Coastal Plain. Enough forage is not always present to provide for the energy requirements of the animal, but field observations support the conclusion that other factors are also limiting. For example, in maintaining herds in the Hoffman Forest, copper was included in the mineral mixture, and the evidence, while not conclusive, indicated that this element was beneficial. Since phosphorus, copper, and iron are low in the youngest terraces (Pamlico and Talbot) and tend to increase as the age and elevations of the terraces increase, it would seem reasonable to conclude that they may also be limiting in these younger terraces.

Conversely, the disappearance of acute deficiencies in cattle on the Sunderland and higher terraces is associated with a reduction in the proportion of poorly-drained soils supporting a forage low in cobalt, and with small but significant increases in the iron and copper content of vegetation. All of these changes are small, but the overall effect may be sufficient to influence the balance between an acute nutritional deficiency and a subacute one that is not readily recognized. Experience in other areas has indicated that this balance is a very delicate one in which the usual animal variation also plays an important role.

Summary and Conclusions

1. A study has been made of the factors influencing the mineral nutrient content of native forages in the Coastal Plain of North Carolina. Emphasis was placed in this study on areas where nutritional troubles in cattle may be due to deficiencies of cobalt in the forage.

2. Physiographic regions, as represented by abandoned marine terraces in the Coastal Plain, were used as a basis for a systematic sampling of native forages. Further subdivisions were made on a basis of three geographical locations or regions transecting the terraces from the coast to the Piedmont Plateau along main drainage lines. Soil drainage conditions were utilized for a third subdivision. Three randomly-chosen locations containing a well-drained and a poorly-drained soil were sampled in duplicate in each location. In all, 288 samples were taken.

3. Two plant species were selected in each area. Leaves of

Arundinaria tecta, a reed or cane, and a common forage for cattle throughout the year, were sampled on the poorly-drained soils. Immature leaves of *Andropogon virginicus* (broomsedge) were sampled on the well-drained soils. Comparative studies of the composition of both these species selected from poorly-drained soils also were made at twenty-three locations throughout the Coastal Plain.

4. Chemical analyses of the forages were made for cobalt, copper, iron, manganese, and phosphorus. Analyses of variance in the data were computed for each factor considered in the study.

5. Leaves of *Arundinaria tecta* (reeds or cane) were higher in copper and lower in iron than was the above-ground portion of *Andropogon virginicus* (broomsedge). No species differences were found with respect to cobalt, manganese, or phosphorus.

6. The content of cobalt was greater in vegetation from well-drained soils, while the converse was true for iron. The copper, manganese, and phosphorus contents of the vegetation were not influenced by drainage conditions.

7. Important variations in iron and manganese occurred in relation to geographical position. Thus, manganese was significantly higher in the southern part of the Coastal Plain. It is suggested that this difference in manganese can be accounted for by a difference in the sources of parent material. No explanation for a north-south variation in iron content is available.

8. The content of all elements except cobalt changed significantly in relation to the marine terraces on the Coastal Plain. There were definite increases in iron and manganese with decreases in age and elevation of these terraces. Copper and phosphorus content showed significant maxima at intermediate elevations, but there was also a small overall increase of these elements as the age and elevation of the terraces increased. A similar but non-significant trend was apparent in the cobalt data.

9. It is concluded that acute symptoms of nutritional troubles in cattle due to deficiencies of cobalt, copper, and other elements may be associated with (1) such factors as the relative areas of poorly-drained soils supporting a vegetation low in cobalt, and (2) small

but important increases in native forages of iron, copper, and other elements in direct relation to the age and elevation of the marine terraces on the Coastal Plain.

Acknowledgments

The authors wish to acknowledge the important contributions of Dr. Branch Moore of Kinston, North Carolina, whose accurate diagnoses of mineral deficiencies in cattle were basic to this study. Professors W. D. Lee of the North Carolina State Agricultural College and J. E. Foster, now of the University of Maryland, also gave valuable assistance. Most of the chemical analyses were made by Richard L. Gregory and Clayton J. Morris.

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DISCUSSION

DR. GORDON ELLIS: I would like to comment on the values for copper that Dr. Beeson has found in North Carolina vegetation. While these values are not extremely low, it seemed worthwhile to examine animals in this region. This was done in collaboration with Dr. J. Weybrew, Dr. C. D. Grinnells, and others at the North Carolina State College at Raleigh. During February, 1949, 79 animals (bovine), very often the family cow which received very little or no feed other than local browse and ground corn cobs and husks grown locally, were examined. With a few exceptions, they were in poor condition. No gross symptoms of copper deficiency were noted. Analyses made on these animals gave the following results: Hemoglobin, 11.4 g./100 ml. blood (54 animals), which is somewhat higher than the value for the college herd at Raleigh, which in a previous study was found to be 9.9 g./100 ml. blood. The serum copper was 73.6 μg./100 ml. (60 animals), a value which could not be considered at all abnormal. The copper content of dry, fat-free hair was 9.7 μg./g. Our thought in analyzing hair was that under field conditions where the feed supply is variable, hair copper might prove to be a more reliable index of the copper status of the animal than would serum copper. The coefficient of variability for the hair copper was somewhat lower than for the serum copper. No correlation between hemoglobin level and serum or hair copper nor between hair and serum copper was found.

While these results indicate that copper deficiency in cattle is not a problem, at least of any serious proportions, at the present time, the low values found in the vegetation would suggest that this and similar areas should be kept under observation.

A SUMMARY OF
THE SYMPOSIUM

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INTRODUCTION

Because copper is an essential requirement for plants and animals alike, because it is known to be characteristic of a number of metalloprotein enzymes, because it is demonstrably deficient in certain types of soils, and finally because it was among the earliest micronutrients to become known, it was chosen for the subject of the first symposium of the McCollum-Pratt Institute to be devoted to a particular trace element. The papers presented at the symposium exemplify, first of all, the two chief methods of attacking experimentally the problem of a micronutrient's role in metabolism, namely, (1) the study of the effects produced by a deficiency of the elements, in the present case copper, and (2) an analysis of enzymes, first to determine which ones are metalloprotein in nature, and thereafter to determine precisely which of the latter contain the element in question. Whereas the first of these approaches would initially appear to be easier, that approach becomes more and more difficult whenever, as with copper, one must deal with an element so widely distributed in nature and so exceedingly difficult to eliminate from the growing medium, whether that be soil or nutrient solution. On the other hand, this type of study may readily throw light on the interactions of one micronutrient with another, a relationship which has proved to be highly important for copper with respect to iron, molybdenum, and perhaps other elements. The second, or enzymological, approach offers a quicker, less devious avenue to a knowledge of the exact role played by copper in plant and animal metabolism. It should be much easier to start with an enzyme that has been isolated and to derive its function and its effects, than to start with an assortment of symptoms produced by a nutrient deficiency and thence to arrive at a biochemical understanding of its causes. That the truth of this generality is fully appreciated by workers in the field of micronutrients is evident from the preponderance of contributions to this symposium which deal, at least in part, with enzymatic relationships.

In addition to papers considering the effects of copper deficiency or the enzymatic functions of copper, a number of other contributions, particularly those by J. E. Dawson, Davis, Trumble, Beeson, Cunningham, and Marston, have added to our appreciation of the relationship between soil and copper nutrition, and have brought out the interrelationships between copper and other micronutrient elements. The particular relationship between molybdenum and copper has been further advanced through the use of radioactive isotopes of these metals, a technique which is amply discussed by Comar in his contribution to the symposium.

Establishment of Copper as a Micronutrient

The list of trace elements found to be necessary, first for plants and then for animals, has lengthened rapidly in the past two decades. Among the earliest of those to be established as essential was copper. In 1927, Bortels demonstrated that copper is necessary for *Aspergillus niger* to form black spores. In the absence of copper the spores are brown and the yield is reduced 50 per cent. Many other fungi exhibit the same requirement. In 1931, Anna L. Sommer, working at the University of California, grew plants in nutrient solutions from which the copper impurities had been removed by refined methods and for which all the water used had been redistilled in pyrex or quartz. Almost simultaneously, Lipman and MacKinney showed that barley will not form seed without copper. These experiments demonstrated that copper is an essential trace element for several species of plants, and further work has led to the general conclusion that it is necessary for all. Just two years later B. Sjollema demonstrated that a wasting disease of cattle in Europe comes from a copper deficiency in the forage, and he thus established the essentiality of copper for an animal species. The study of the " falling disease " and of " stringy wool " in Australian sheep added a second species.

Localities where these deficiency diseases of plants and animals are prevalent are distinguished by two special types of soil. In the first, peat soils or soils with a high humus content, plenty of copper is present, but it is tied up by the organic matter and rendered largely unavailable to growing plants and animals. In sandy soils, on the contrary, there is an actual lack of copper.

Another significant discovery in this field had already been made by Waddell, Elvehjem, Steenbock, and Hart at the University of Wisconsin in 1929. Rats which had been made anemic on a diet of whole milk could not recover when iron alone was added to their diet, but they recovered rapidly when minute amounts of copper were also included. Copper was thus proved to be in some way important for the synthesis of hemoglobin. The exceedingly small amounts of it required, a few parts per million, hinted that it might be a part of some enzyme.

In 1938, Kubowitz found that certain enzymes do contain copper. These enzymes are oxidases of the type which catalyzes the reduction of molecular oxygen. Their substrates are phenolic substances or ascorbic acid. The copper enzymes occur very widely. Like most other purified metalloprotein enzymes, they never possess any except their one characteristic metal.

THE PROPERTIES OF COPPER WHICH AFFECT ITS FORMATION OF ORGANIC COMPLEXES

All discussions of metabolism require a physicochemical background. For the present symposium this was supplied by Corwin, who discussed the nature of the forces underlying the formation of copper complexes. The polarity of the copper atom is the appropriate starting-point for such considerations, for it determines the place of copper in the Periodic Table, the size of the copper atom, the angles between its bonds, the strength of its chemical binding, and its mutual affinities with other atoms. The atomic radius of an atom is a function of its electrostatic positive and negative charges, and its magnitude in turn determines the number of groups which can be placed around the central atom in the formation of complexes. The copper atom is of moderate size and could always accommodate four, and usually more, groups about it; as many, indeed, as its other properties will permit. This provides an important degree of freedom in the formation of copper complexes.

The shape of the complex is also significant. The bond angles of cuprous complexes coordinating two groups are probably in all cases 180°, whereas those with four groups, like carbon complexes, have bonds at angles of approximately 109.5°, making the complex tetrahedral in shape. On the other hand, cupric complexes, whether possessing 4 or 6 covalences, are octahedral, with 90° angles between all bonds. Thus when copper is substituted for carbon, the larger size of the copper atom closely balances the difference in the bond angle between 90° and 109.5°, so that copper forms very stable 5-membered rings, as in the copper-ethylene glycol complex.

The chemical affinities of nitrogen, oxygen, and sulfur for copper vary in relative magnitude as one goes from air to solutions. For example, in the dry state sulfur's affinity for copper is less than that of oxygen, and this relationship holds in other media of very low dielectric constant, such as lipoids. In aqueous solution, on the contrary, the affinity of oxygen for copper diminishes relatively more, because of ionization, than the affinity of sulfur or nitrogen for copper. Biological systems are complex and both aqueous and fatty. Consequently they must show, with respect to the complexing ability of copper, a variance that will depend on the nature of the site where the complexing reaction takes place. If this is fatty, oxygen will show greater affinity for copper; but if it is aqueous, the order will be sulfur, then nitrogen, and last oxygen.

The complexing ability of copper also depends upon the number of groups which can surround one copper atom. From the rare gas rule it follows that " the maximum covalence of an element is determined by the number of electrons required to fill its orbits to that of the next higher rare gas." For Cu, this is krypton; and the deduction follows that the maximum covalence of Cu is four. For the cuprous state, this rule holds very well, complexes with 2, 3, or 4 coordinated groups being frequent, but none higher occurring. The cupric complexes should, by the rule, be limited to a maximum of 5 groups. Actually, cases with 2 or 4 groups are frequent, those with 3 or 5 groups rare; but some with 6 groups occur. These 6-group complexes indicate exceptional properties of Cu worthy of special investigation.

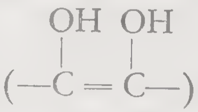
Finally, Corwin discussed the tremendous increase in stability of compounds which is brought about by chelation, that is, the formation of double or multiple bonds to the same organic molecule.

All of these factors are combined to give great stability to such copper complexes as the copper porphyrins. The hole in the center of the porphyrin ring is just the right size for the copper atom; the four N atoms are bonded at 90° angles, just right for copper; the N atoms, specifically, show great affinity for Cu in aqueous solution; and finally, the quadruple chelation makes a remarkably stable complex. The balance of forces may be altered in innumerable ways through the capacities of various proteins to chelate or otherwise modify the interacting factors. To quote Corwin's concluding sentence: "This interplay of forces gives richness and variety to the functions which can be achieved by any given chemical element and is undoubtedly responsible for the ever-increasing number of uses which we are finding for elements such as copper in metabolic processes."

Copper exists as several isotopes with the same atomic number but of different weights. Of these, the radioactive isotope Cu^{64} offers the possibility of applying tracer techniques to the study of copper metabolism. In a most helpful discussion, Comar has weighted the usefulness and the difficulties of using Cu^{64} and also Mo^{99}, which is of interest here because of its metabolic interactions with copper. The brief half-life of Cu^{64} (12.8 hrs.) limits its use to short-term experiments. The high cost of the cyclotron-produced isotope limits it to small-scale experimentation. The great loss of sensitivity when the activity of a solution rather than a dry film is measured is an obstacle; and the methods used for preparing and concentrating the samples are critical. The dosage must be calculated with the need for obtaining a measurable activity in the final sample and also with the organism's normal tolerance in mind. Ideally, one should employ a tracer dose, or at least a dose no larger than the organism's normal intake of the substance, and yet it should be one that would have, in a sample of convenient size, a level of activity with a count at the time of measurement several times that of the background. The toxicity of copper cannot be ignored, nor the dependence of the toxicity on the mode of administration. The toxicity for a rabbit, for example, is at least 40 times as great for intravenous administration as when the copper is ingested with food. In contrast, the use of Mo^{99} is not fraught with quite such difficulty, since its half-life is much greater, its toxicity for animals is less, and its absorption more.

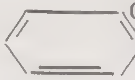
THE COPPER-PROTEIN ENZYMES

The enzymes known to contain copper include oxidases of (a) ascorbic acid, (b) p-phenylenediamine, and (c) polyphenols, such as tyrosine. In these enzymes the copper is firmly bound to a specific type of protein and cannot be removed by simple dialysis. When removal of the copper is accomplished (for example, by cyanide treatment), the enzymatic power is lost. Knowledge regarding the nature of the copper-protein bond and the size, shape, and electrical properties of the copper-protein molecules is limited to the hemocyanins, ascorbic acid oxidase, and polyphenol oxidase.

The characteristics of the enzyme ascorbic acid oxidase, including its likeness to and contrast with tyrosinase, have been ably set forth by C. R. Dawson in his contribution to the symposium. Most remarkable is their completely distinct specificity as to substrate, in spite of their complete similarity in copper content and protein properties. Dawson has emphasized that both ascorbic acid and tyrosine contain an ene-diol group  $(\text{—}\overset{\text{OH}}{\overset{|}{\text{C}}}=\overset{\text{OH}}{\overset{|}{\text{C}}}\text{—})$ which is involved in the oxidation occurring, and which is adjacent to a carbonyl group and ring structure. Moreover, ascorbic acid oxidase, tyrosinase, and all other copper enzymes become irreversibly inactivated during the course of the reactions they catalyze. This takes place rapidly and is not a result of any products formed. Since the inactivation is consequently inherent in the nature of the enzyme, the hypothesis that the inactivation results from some " irreversible rupture of the copper-protein bond " is attractive. Dawson has brought forward new evidence bearing on this point, from experiments done with ascorbic acid oxidase obtained in pure form. Crystalline ascorbic acid is markedly decreased in stability by copper, and the activity of purified ascorbic acid oxidase preparations parallels their copper content. That the copper is actually bound in a protein complex and is not free or ionizable has been maintained by Dawson and his colleagues, and the cumulative evidence is now compelling. In particular, Dawson has found that the enzyme in a " resting " condition does not undergo exchange with radioactive Cu^{64}, although it does so when it is oxidizing its substrate. The uniform electrophoretic mobility of different preparations of the enzyme and a similar homogeneity in ultracentrifuge tests alike support the conclusion that the enzyme is a specific copper-protein. The sedimentation and diffusion constants indicate that it has a molecular weight of about 150,000 and contains 6 copper atoms per molecule. Dawson has put forward a highly interesting hypothesis to account for the nature of the enzyme reaction and inactivation. He proposes that the copper is divalent to start with, shuttles reversibly between the divalent and monovalent states during catalysis, and that exchange of the copper occurs only when the enzyme copper is monovalent. The last postulate is a new one, based on the relative bond strengths of the cupric and cuprous states, the former, as Corwin pointed out, being by far the stronger. Experiments with nickel complexes have shown that nickel undergoes exchange only when in the monovalent state, and this makes it seem very probable that copper behaves so too. Finally, since copper is exchanged only in the presence of oxygen as well as ascorbic acid, it seems probable that a ternary complex of oxygen, enzyme, and substrate is formed during the reaction.

The enzyme which acts upon p-phenylenediamine is known as laccase. It is found in the latex of several species of lacquer trees. Arnon has pointed out in his discussion that, besides the above substrate, laccase also oxidizes ascorbic acid directly. Among the copper enzymes he sees a certain lack of substrate specificity, since a number of them, including polyphenol oxidase, can oxidize ascorbic acid. This contrasts with the view of Dawson regarding the specificity of ascorbic acid oxidase.

Among the copper enzymes, polyphenol oxidase, or tyrosinase, as it is also called, has been the subject of most investigation, in both plant and animal metabolism. Mallette has surveyed thoroughly the presence and activity of this enzyme in animals, while both Arnon and Nelson have examined its relation to plant respiration. Enzymes of this type occur very widely in nature, especially in plant tissues which darken upon injury, in numerous invertebrates where they are concerned in the hardening of the cuticle, and also in the melanotic tumors and pigmented areas of the skin or feather germs in vertebrates. Polyphenol oxidase acts on a number of natural substrates besides tyrosine (monohydroxy-phenylalanine) and dopa (dihydroxy-phenylalanine). These include tea tannins, chlorogenic acid, and a variety of protein enzymes, etc., which presumably contain tyrosine.

The activity of the enzyme has been studied extensively in work with monohydroxyphenols, such as p-cresol (HO CH_3), and dihydroxyphenols, such as catechol ( OH OH). Since most preparations exhibit activity toward both of these substrates, the question has been raised whether there are really two enzymes, a cresolase and a catecholase, of distinct specificity. Mallette has dealt particularly with this problem. He comes to the conclusion that tyrosinase is most likely a single enzyme with two characteristic activities. If so, the molecular size of the enzyme must vary with the ratio of its catecholase to cresolase activity, the loss of certain non-copper portions of the molecule diminishing its cresolase activity.

The evidence strongly supports the view that the phenols are oxidized by the enzyme into catechol, i.e., from the monohydroxy to the dihydroxy condition. Catechol is oxidized to the ortho-quinone, and the latter may yield either spontaneously or enzymatically a 4-hydroxy-orthoquinone, which rapidly polymerizes into pigments. If catechol oxidation must occur simultaneously in order for the phenol to be oxidized, then a ternary complex of catechol, enzyme, and phenol is probably formed. The oxidation of tyrosine or dopa follows the same general course. Tyrosine is oxidized to dopa; and dopa is then oxidized to the corresponding quinone, which is rearranged and further oxidized to hallachrome. This is still further oxidized by the enzyme, in either acid or alkaline medium, to one or another of the characteristic black or red melanin pigments. The important feature in all of these oxidations is that the enzyme behaves as a true terminal oxidase, utilizing molecular oxygen, and being consequently comparable to the cytochrome oxidase system.

In such fungi as *Aspergillus*, copper is necessary for the conversion of brown pigment to black in the spores. Free oxygen is also necessary, so that the evidence points to the action of a copper oxidase here too (cf. Steinberg).

Respiration in plants is commonly considered to depend upon the cytochrome system, with cytochrome oxidase playing the role of terminal oxidase. Arnon points out, however, that the cytochrome system is not present in all plant tissues or even in the same tissue at different stages of growth. Nelson also cites the work of Schade to show that only 35 to 40 per cent of the respiration of potato slices in the dark depends on cytochrome oxidase as the terminal oxidase. The cytochrome and polyphenol systems may well be supplementary or complementary in respiration. The objections of Szent-Györgyi, Schade, and others to the view that the polyphenol oxidase system is important in respiration hinge on the extremely rapid oxidation of catechol as well as its toxic effect, and on the inhibition of tyrosinase by carbon monoxide in the light as well as the dark. Arnon, however, questions the validity of the experiments which appeared to show that CO inhibition of respiration is reversible by light. Moreover, other substrates such as protocatechuic acid and dopa are utilized more slowly than catechol and without untoward effects. Nelson reports that recent work done by Walter in his laboratory shows that homogenates of sweet potatoes increase their respiration greatly when catechol is added, but very little when cytochrome C is added. Arnon suggests that the quinones formed by the oxidation of these substrates would be promptly reduced by other respiratory metabolites, and that only when injury interrupted the normal path of respiration would further oxidations occur, producing pigments and bringing about a discoloration of the tissues. This fits well with the findings of Szent-Györgyi and Vietorisz that upon injury the uptake of oxygen is greatly increased, a fact which led them to look upon the role of polyphenol oxidase as that of a protective enzyme that forms products with a tannin action and thus provides a protective coat for the injured tissues. Nelson reports that Rudkin has isolated chlorogenic acid and an unidentified component A from sweet potatoes, and that both of these substances act like catechol tannins and increase the respiration of potato slices.

Arnon goes on to point out that even catechol might serve as an electron carrier, shuttling back and forth in coupled oxidations, since in such a system the catechol would be required only in very minute amounts. Polyphenol oxidase with catechol can oxidize the reduced coenzymes I and II, so that the copper enzyme is linked to the oxidative pathways in the cell as in the following scheme:

$$\begin{tikzcd}
 & & \text{CoI} \arrow[r, dashed] & \text{CoIH}_2 \arrow[dr] & & & \\
\substack{\text{H}_2\text{A}\\ \text{substrates}} \arrow[r] & \text{dehydrogenases} \arrow[ur, no head] \arrow[dr, no head] & & & \text{o-quinone} \arrow[r, dashed] & \text{o-phenol} \arrow[r] & \substack{\text{Cu}^{++}\\ \text{enzyme}} \arrow[d, dashed] \\
 & & \text{CoII} \arrow[r, dashed] & \text{CoIIH}_2 \arrow[ur] & & & \substack{\text{Cu}^{+}\\ \text{enzyme}} \arrow[d] \\
 & & & & & & \text{O}_2
\end{tikzcd}$$

(Solid arrows indicate the direction of electron transfer)

A confirmation of these relationships would greatly clarify our knowledge of cellular respiration. It may lead, in fact, to an appreciation of the role of copper in metabolism that will parallel the recognition of the importance of iron that followed the elucidation of the cytochrome system and its fundamental place in respiratory processes.

It has also been thought that tyrosinase might function as a terminal oxidase in invertebrates, particularly in the insects. Dethier points out that injured tissues of insects do increase their respiration when tyrosine is added; but so far there is no clear evidence that normal tissues do. Neither inhibitors of copper proteins nor phenolic substrates proved to have any effect on the respiration of live pupae of the cecropia moth, in experiments conducted by Sussman.

Thompson has contributed to the symposium a consideration of the relations between the browning of foods and the activity of copper-proteins. The Maillard, or browning reaction, was found during World War II to depend on the presence of trace amounts of metals, particularly copper. It has been shown that in milk the copper is not free but is bound to protein, particularly at higher pH values. Baudisch found a simple reaction which is virtually limited to copper among the metals, and which he postulated as the cause of browning in foods. In this so-called B reaction, cuprous nitrosyl (CuNO) reacts with compounds having an ethylene group in a ring structure, e.g., benzene, to produce an o-nitroso-phenol. This product reacts with cupric ions to form a stable, deep red, complex copper salt. The relation of this B reaction to the copper enzymes is to be seen in the fact that among the substances which react with casein in the presence of copper to produce browning are such known substrates of the copper enzymes as ascorbic acid, catechol, and phenol. Other substances found to react in this way also have an ethylene group in a ring; but such substances as glucose, although they are involved in other types of browning, do not give browning with the copper-casein. It was also found that a number of other proteins can replace casein in the reacting system, but casein hydrolysate and various amino acids cannot. Whole proteins thus appear to be essential to the complex. Copper-albumin differs from copper-casein, however, for it browns rapidly with aromatic compounds but only very slowly with ascorbic acid. In general, these copper-protein complexes responsible for the browning of food lack that high degree of specificity characteristic of the copper enzymes.

The investigations on the browning of foods have not yet established the nature of the copper-protein complex, but they do indicate that a greatly increased browning activity results when the copper is not free, but is in some way bound. Practical values may be expected to result from these findings. One is the realization that containers with a high metal content or contamination are likely to promote swift browning of the foods they contain. More striking is the effect of a high copper concentration in preventing spoilage. Thompson tells of various samples of milk, treated with a copper-casein-catechol complex, that quickly turned dark brown but neither soured nor became putrid in the space of 15 months. The same experience occurred with milk having a high natural content of copper (0.15 p.p.m.).

COPPER METABOLISM OF INVERTEBRATE ANIMALS

Few symptoms of copper deficiency have ever been seen in invertebrates. In fact, as Dethier has pointed out in his concise review of this subject, the invertebrates, especially the arthropods and mollusks, surpass plants and vertebrates alike in the accumulation and concentration of copper. Yet apart from the role of the respiratory pigment hemocyanin, the functions of copper in invertebrate metabolism still remain obscure.

In marine invertebrates, copper absorbed from the food becomes concentrated in particular organs, especially the digestive and storage organs and the gonads. Oysters sometimes become quite green from its accumulation in certain ameboid cells beneath the surface epithelium. This is interesting, but its significance is a mystery. There are signs, however, that the distribution of copper in various organs is related to the maintenance of a satisfactory copper balance as a steady state. In insects, for example, the alimentary canal and the malpighian tubules, the one concerned with the absorption of copper and the other with its excretion, are each rich in copper; but the gonads are relatively poor. In the midgut of the larva of the sheep blowfly, *Lucilia cuprina*, and of other flies, there are cells with special copper-absorbing powers. It is striking that although copper is abundant in the blood of insects, yet their main supply of copper is not in the blood, as in those invertebrates that possess hemocyanin, but in the tissues. The blood carries much less than would be expected on the basis of its proportional weight. From the fact that the copper which reaches the tissues can no longer be demonstrated by the staining reactions commonly used for copper, it is apparent that the copper becomes bound. In the blowfly, about one-third of its total copper enters into organic combination.

The known copper-protein enzymes of invertebrates belong to the tyrosinase group, the enzymatic action of which has already been summarized. These enzymes perform a variety of functions in the life of the invertebrate organism. They harden the fresh cuticle after a molt, or toughen the organs of attachment formed when a free-swimming larva settles down and is transformed into a sessile adult. They help to form the characteristic pigments, and are involved in the formation of pigmentation patterns. In the squid or octopus, they produce melanins for the ink; and in many invertebrates, they are concerned with the hardening and blackening of the blood upon injury. The tanning reaction is best known. In this reaction, a network of proteins is produced through the formation of many covalent bonds between collagen proteins, through the action of a quinone. Besides being of fundamental importance to arthropods in the hardening of their exoskeletons, this reaction has been reported to occur in protozoans, roundworms, flatworms, annelids, and mollusks.

Whether or not copper has any effect on the growth and metamorphosis of invertebrate animals remains quite uncertain. Efforts to produce effects by a copper deficiency or by a copper excess have usually given only negative results. Among marine forms with free-swimming larval and sessile adult stages, such as hydroids, barnacles, and ascidians, copper is among those substances that provoke attachment of the larva and its transformation to the adult.

Higher concentrations of copper are well known to be toxic to many organisms. Yet even today nothing certain is known regarding the physiological mechanisms whereby Bordeaux mixture kills insects or antifouling paint acts on barnacles. A clue might lie in one of the most remarkable discoveries. In the citrus orchards of southern California, strains of scale insects which had a high resistance to cyanide fumes were found to contain only 10 to 20 per cent as much copper as the susceptible forms. Since the difference between strains was shown to be hereditary, it seems that mutation can give rise to biochemical differences in copper metabolism that in turn may greatly affect resistance or susceptibility to other agents.

Of the several copper proteins, hemocyanin has been known longest. Like most of the others, it too has a characteristic blue (or blue-green) color. It occurs in certain groups of the phyla Arthropoda and Mollusca, being limited in the former to the horseshoe crab, the scorpions, and the larger decapod crustaceans, and in the latter to the cephalopods and some gastropods. Near relatives of gastropods with hemocyanin may have hemoglobin instead, and in fact the two oxygen-carrying pigments may occur in different tissues of the same animal (*Busycon*). Such a scattered distribution implies that hemocyanin has arisen independently in evolution a number of times, a conclusion borne out by the existence of small but characteristic differences between the hemocyanins of different species. In having arisen independently several times in the course of evolution, as well as in its function as an oxygen carrier in respiration, hemocyanin resembles hemoglobin. Unlike the latter, however, hemocyanin is always dissolved and never aggregated into corpuscles. The units which contain a single atom of copper have about twice the molecular weight of an analogous unit of hemoglobin. Because the *functional unit* combining with one molecule of oxygen requires two atoms of copper, it has a molecular weight (50,000 to 74,000) approximately four times that of a functional unit of hemoglobin, in which one atom of iron combines with a molecule of oxygen. Many functional units of hemocyanin are aggregated into a single giant globulin molecule up to 17 mμ in diameter, a size exceeded only by the viruses.

Redfield has emphasized how the work on hemocyanin has followed the pattern of the earlier advances in our knowledge of hemoglobin. Just as hemoglobin turned out to be only one among many iron heme compounds, so hemocyanin is turning out to be only one of a number of closely related copper proteins; and just as the study of hemoglobin opened the way toward an exploration of the other iron proteins, so hemocyanin should " serve as a useful prototype for the understanding of other copper compounds of biological interest." All the copper proteins have about the same copper content as hemocyanin, and the latter has some phenolase activity. Hemocyanin resembles polyphenol oxidase in a number of ways, such as its combining power with oxygen. After treatment with cyanide, copper can be restored to hemocyanin only in the cuprous state. This is a forcible reminder of the theory advanced by Dawson for ascorbic acid oxidase, that the exchange of copper occurs only when the copper is monovalent.

The primary problem with respect to the hemocyanins is that of the nature of the prosthetic group, analogous to heme, with which the copper combines. The copper is certainly more loosely held in some substances of this class than in others. Various derivatives of the hemocyanins containing high percentages of the copper originally present have been prepared. None of these has shown any evidence of a porphyrin structure, although some of them did contain pyrrol nuclei. This means that the once widely held opinion that hemocyanin has a porphyrin structure like hemoglobin and chlorophyll is probably mistaken; yet there is a more distant chemical relationship. According to several studies, the prosthetic group of hemocyanin is a polypeptide containing, or closely associated with, two divalent acid- or base-binding groups. But the basis of hemocyanin's specificty remains unknown.

Redfield himself has investigated the oxygen dissociation of hemocyanin. The dissociation curves obtained for the hemocyanins from different species show that in certain species and at a given pH the prosthetic groups act " independently, as though each were a separate molecule." In other forms or at other values of pH, the prosthetic groups form aggregates of two or four; while in still other species probably two or more of these states of aggregation may exist together. An electron micrograph of hemocyanin from the gastropod *Busycon* appears to show these various states of aggregation, and so affords a remarkable visible confirmation of differences in chemical activity. Much may be learned from the further analysis of the differences in degree of aggregation, in the ultraviolet absorption spectra, and in other properties of the hemocyanins found in different species.

The Copper Metabolism of Vertebrates

Marston has reported the results of studies in Australia of the relation between the copper status of sheep and enzyme metabolism. Simple copper deficiency leads to changes in the wool of sheep long before the animals exhibit the slightest other signs of ill health. The fleece becomes stringy on account of inadequate keratinization, and in a black sheep the follicles not only lose their capacity to impart a crimp to the wool but also their ability to synthesize melanin. A staining test for free thiol (–SH) groups shows that these groups are abundant in the hairs of a copper-deficient animal for a distance about ten times as far from the constriction in the hair follicle as is the case in normal hair. Upon dosing the animal with copper, this region of free –SH groups becomes normally restricted, in just a few hours. It therefore seems that copper is " responsible for catalyzing the oxidative closure of the thiol residues of the prekeratin fibrous protein to the disulphide linkages of keratin. The rate at which this reaction proceeds is a measure of the copper status of the sheep."

The cells of the hair attain their elongated shape by being squeezed through a cone-shaped constriction of the follicle. At the same time, apparently, the fibrous protein molecules become aligned so that later, when they are rapidly oxidized to keratin in the presence of the copper enzyme, they are fixed in the same position. The slower oxidation that occurs when copper is deficient must allow the protein molecules to become disoriented. This is most interesting, for it reveals an entirely new aspect of the enzymatic powers of the copper oxidases, and yet one that is somehow closely connected with the better known tyrosinase type of activity which is also being carried on in the same hair follicles. Cartwright has confirmed this increase in rate of oxidation of –SH (glutathione, cysteine) in the presence of copper.

The discovery that the rate of oxidation of cysteine to cystine is dependent upon the presence of iron was one of the first bits of evidence which enabled Warburg to establish the importance of the cytochrome system in respiration. Now comes this parallel evidence from Marston's work to lend credence to the theory, proposed in this symposium by Arnon and others, that the copper oxidases represent a system alternative to the cytochromes, supplementing or in some cases supplanting the latter in the terminal stages of respiration that enable molecular oxygen to be utilized.

On the other hand, it has been demonstrated (cf. Cartwright) that in rats copper plays a role in the formation of cytochrome A, cytochrome oxidase, and catalase. If this role is general, it may be that

Marston's findings are based on an *indirect* effect of the copper enzymes upon the rates of oxidation of –SH groups, an effect mediated through the interrelation of the copper enzymes and the cytochrome system.

Experiments using the isotope tracer technique with Cu^{64} have shown that a considerable exchange and accumulation of copper goes on in the higher animals. Dogs and rats, after ingesting copper, show a rapid rise in the copper in the blood plasma, and later the copper is to be found in the red cells. The liver, kidneys, and bone marrow accumulate most copper. Copper is, however, not retained very long. Meanwhile, it provokes a higher activity of cytochrome C oxidase and of blood cell formation. Comar, who has reported these findings, has worked with various colleagues on cattle, which have a greater sensitivity for a copper deficiency than non-ruminants. When copper is ingested with the food, about three-fourths is found in the feces and about 3 per cent is excreted in the urine within 5 days. Intravenous administration is approximately ten times as effective as ingestion, in terms of the concentrations of copper found in the tissues, and only about 6 per cent of the dose is lost in 5 days. The liver is clearly the chief storage organ, although the kidneys show a high concentration and in other species may exceed the liver in copper. Cartwright states that in an adult human being there are from 100 to 150 mg. of total copper. Most of this is deposited in the muscles, bones, and liver, the highest concentration, although not the greatest amount, being found in the liver.

Because of the already mentioned relation of copper to the synthesis of hemoglobin, it is surprising to find that in cattle there is no great concentration of copper in the actual centers where the red blood cells are formed. The red bone marrow has only a medium concentration, although the intestinal mucosa, which possesses an important hemopoietic activity for white blood cells, shows a rather high concentration of copper.

Although little is known about the site of absorption of copper in the human digestive tract, there is evidence (cf. Cartwright) that the kidneys are not an important route for the excretion of copper, and that most of it is excreted in the bile or directly into the intestine. This type of excretion may be characteristic of invertebrates as well as vertebrates, for in the discussion following Dethier's paper Poulson brought out the fact that the malpighian tubules of insects show very little correlation between the amount of copper fed and the intensity of staining for copper; whereas the midgut cells show a very high correlation. Poulson therefore held the latter to be the chief organs of accumulation and excretion, although the excretion might be chiefly by storage.

Copper in the circulation has been studied extensively in man and other mammals. In man, according to Cartwright's summary, plasma copper ranges from 92 to 123 μg. per cent. There is normally about two-thirds to three-fourths as much copper in the red cells as in the plasma, but leucocytes have much less. Individuals may differ in plasma copper by a factor of 3, but any one person stays within a range of ± 30 per cent over short periods. Very little is known about the factors which control the plasma concentration of copper. Its transport is apparently in loose combination with plasma proteins, but only a negligible amount of the plasma copper is in transport at any time. Most of the copper seems to be combined with the α_2 and α_3 globulins, for these show a high correlation with the copper concentration of the serum. A new blue-colored copper protein, caeruloplasmin, has recently been isolated among the α_2 globulins. It has eight atoms of copper per molecule (weight 151,000) and contains 90 per cent or even all of the serum copper. Its function is at present unknown. It will be most interesting to compare it with hemocyanin.

Maintenance of the copper balance requires in human adults an intake of 2 mg. per day or more. More is retained as the intake is increased, up to 8 mg. per day. For children about 80 μg. per kg. of body weight is the usual requirement. Even mediocre American diets more than supply such amounts. According to Marston, sheep are more sensitive to a low intake of copper. Sheep which get as little as 2 to 4 mg. per day become depleted in about half a year. The concentration of copper in the blood has already dropped to one-third the normal level and is about 1.0 μg. per ml. when symptoms of defective keratinization show up in the wool. This level may be reduced to one-tenth as much, parallel with a slowly progressing hypochromic anemia.

An age difference is regularly found. Calves are born with four to eight times as much copper in the liver as adults. Because of the very low copper content of milk, this high level is reduced during the suckling period, although calves 1½ months old still show a higher ability to accumulate copper than older animals, according to Comar's studies. Cartwright states that the same relations hold true for the human fetus. The copper concentration of its liver is five to ten times that of an adult, increasing to a maximum at term. It decreases steadily after birth, but is still higher in a one-year-old than in an adult. Between the ages of 5 and 15 years the adult level is reached. Marston has likewise emphasized the increased significance of the copper nutrition for lambs, compared with sheep. Iron is " concentrated in the fetal liver irrespective of the iron-status of the ewe," but the copper in the fetal liver or in the milk directly reflects the copper-status of the ewe. When the concentration of copper in the ewe's liver drops below 10 μg. per g. (dry weight), the lambs born always suffer from nervous manifestations characterized by a spastic paralysis of the hind legs. These nervous disorders range, depending on the severity of the copper deficiency, from complete paralysis, fatal in a few days, to a bland ataxia which is manifested in lack of coordination of the hind legs, staggering, and a stiff gait. The lambs do not seem to lose appetite, but even after an apparently complete recovery, which is possible in mild cases if copper is fed, there must still be lesions in the nervous system, since ataxia reappears when the animals are startled. This disorder always appears in the lambs if their mothers show any symptoms of copper deficiency, and even (in England) sometimes when they do not.

The pathological effects of copper deficiency have not been studied sufficiently to allow a clear picture to be drawn, even for a single species; and the effects are different for different species. In the Australian lambs with ataxia, the neural lesions are visible as a demyelination of the nerve fibers of the large motor cells of the lower spinal cord which serve the hind legs. It is surprising to learn that in New Zealand, according to Cunningham, although this nervous disorder of Australian lambs described by Marston occurs in essentially the same form, nevertheless the stringy wool seen in copper-deficient Australian sheep has not been found. On the other hand, osteoporosis develops in the New Zealand lambs to such an extent that they may suffer fractures from minor falls. This condition, which was not reported to occur in the Australian lambs, seems to result in New Zealand from a copper deficiency even before there is any ataxia among the lambs.

The effects of copper-deficiency in the New Zealand cattle, mainly Aberdeen-Angus and Hereford, parallel the symptoms in the Australian sheep. The animal is anemic, its coat is poor and stringy, and the color may be considerably bleached. Ataxia centers in the hind limbs. But in addition these cattle, like the New Zealand sheep, develop an osteoporosis that leads to fractures of their bones. Cattle in Florida, described by Davis, react similarly to copper deficiency. The coat grows rough and fades, the animal becomes anemic, and its bones grow weak and liable to fracture. In the young calf, the symptoms resemble rickets; in older animals, osteomalacia. But an abnormal gait was noticed only occasionally, and definite ataxia not at all!

In human beings the effects of copper deficiency have practically never been observed; but Cartwright has pointed to a great many pathological conditions in which the copper of the body is increased or decreased. For example, copper increases in both blood plasma and cells in acute and chronic infections and in leukemia; but in pregnancy or cancer it increases only in the plasma. Copper in the red cells increases without any accompanying change in the concentration in the plasma, in iron deficiency anemia. In pernicious anemia, it is the macrocytes that show a significant gain. In the storage organs, especially the liver, copper is increased in malignant disease, tuberculosis, cirrhosis of the liver, and other chronic diseases with associated anemia, but on the contrary it is decreased in simple nutritional anemia. Nothing is understood at the present time of the meaning of such changes in copper concentration in disease, except for the cases of simple depletion. The exploration of this field ought to prove highly fruitful.

INTERRELATIONSHIPS OF COPPER METABOLISM WITH MOLYBDENUM AND OTHER ELEMENTS

Much has already been said regarding the relationships between the metabolism of copper and of iron. In the utilization of iron, copper is of prime importance. Evidence has been rapidly gathering that molybdenum plays a corresponding role in the utilization of copper. There is not sufficient evidence at the present date, according to those discussing the matter, to regard molybdenum as an essential trace element in animal nutrition. That may come. There is, however, striking proof of the indirect importance of molybdenum. Marston has described the intestinal disturbances which arise in cattle on the " teart " pastures in Somersetshire, where the cattle get a high concentration of molybdenum in their forage. This can be cured in just a day or two by feeding the cattle with *copper.* This fact has led to much speculation about the effect of molybdenum upon the copper metabolism of animals. Marston was unable to find that added molybdenum ever brings on the typical nervous symptoms of copper deficiency in normally pastured sheep; or that it will prevent the toxemic jaundice due to copper accumulation which occurs in sheep on pastures too high in copper. New evidence, however, supports the theory that molybdenum can deplete the copper concentration of the tissues and seriously interfere with the copper metabolism. Although no pathological symptoms appeared in sheep on normal pasturage when they were fed additional molybdenum, it was found that while their plasma copper remained normal, the copper stored in the liver was definitely decreased at the time when the available copper in the pasture became seasonally lowered, and the animals could not replenish their copper as fast as untreated animals when the available copper again increased. In contrast, sheep on deficient, calcareous tracts reacted in quite the opposite way to added molybdenum. The depletion of their liver copper was lessened, their plasma copper remained high, and nevertheless *aggravated symptoms of copper deficiency were precipitated.* Added copper will cure the effect in a spectacular way. This can only mean that the copper present in the tissues must under these conditions be bound in some way. It is not physiologically effective. "Molybdenum apparently tends either to fix copper in a form that is not utilizable by the tissues, or to antagonize, by hindrance, the copper-containing enzymes." The same observations have been made experimentally on rats.

Cunningham has described a similar seasonal type of copper deficiency in New Zealand cattle. It is associated with intestinal disturbances ("scouring") and is known as "peat scours." It occurs only on pastures that are high in molybdenum, although not so high as in the teart pastures of Somerset. A low concentration of copper and a high concentration of molybdenum in the liver is characteristic of peat scours, and extra copper in the diet not only cures the disease but reverses the concentrations of copper and molybdenum in the liver, thus proving that a reciprocal relation exists between them. Ataxia in sheep on the same type of pasturage may also be related to molybdenum, but this remains to be shown. The similarity of the peat scours to the conditions produced in Marston's experimental sheep on normal pasturage is so striking as to need no emphasis. Cunningham has produced the same effects experimentally too. By feeding extra molybdenum at about 60 p.p.m. to young cattle, the intestinal disturbances, low liver copper, and brittleness of the bones leading to fractures can be brought on. Sheep fed molybdenum also show a steady drop in liver copper, but so far no ataxia has been produced in the lambs in spite of their grossly depleted copper.

Shifting attention to Florida again, we find that Davis has related the defects in bone-building found there in cattle with symptoms of copper deficiency to the effects of molybdenum on the utilization of copper and indirectly of phosphorus. The amount of phosphorus in the forage was not low, but in the presence of a low copper and a high or even moderate molybdenum content (1 to 26 p.p.m.), typical symptoms of rickets developed in calves, along with liability to incur fractures, anemia, low hemoglobin values, changes in the coat—in fact, all of the typical symptoms previously described. The phosphorus and calcium levels in the blood seemed to be quite normal or above, so the fault in bone building did not lie there. The symptoms could be eliminated speedily by feeding the animals with copper sulfate. As soon as this was stopped, relapse would occur. In older animals, the changes are slower but equally characteristic. Experiments with rats and swine have helped to clarify the picture. As long as copper is high or even moderate in amount in the food, excessive molybdenum does no harm. But when the animals are on a low copper diet, added molybdenum accumulates in the liver and aggravates the copper deficiency. In the rats, this combination appears to lead to stunting of the young, but swine appear to be very resistant to molybdenum, and can tolerate 1000 p.p.m. in the rations for 90 days or longer without ill effects. This reminds us (cf. Cunningham) that in New Zealand, on the same pastures as the cattle or sheep which manifest the symptoms of copper deficiency, horses remain quite healthy.

Comar and Davis have carried out studies with radioisotopes to explore more fully the interrelations of copper, molybdenum, and phosphorus in metabolism. By using P^{32}, it was learned that the excretion of phosphorus in cattle is not altered in amount by high molybdenum, high copper, or both together, but the pathway is shifted from the kidneys to the intestines, particularly when both high copper and molybdenum are combined. On the other hand, in rats the effect of these elements on the phosphorus metabolism is to slow down its excretion, and not to change its route.

When Mo^{99} is fed to a steer, it is absorbed fairly well. Following feeding or intravenous injection of this isotope, its distribution in the tissues resembles that of copper. When Cu^{64}, Mo^{99}, and P^{32} were used together in various combinations on rats, it was found that copper does not affect the uptake of phosphorus, but that molybdenum increases it to a very considerable extent. However, copper and molybdenum together *reduce* the uptake of phosphorus. The accumulation of phosphorus is decreased by prior feedings of molybdenum, and the accumulation of copper is decreased by simultaneous administration of either phosphorus or molybdenum, or both, with the copper.

The toxicity of molybdenum is diminished by copper. This is hardly due to the simple formation of a copper molybdate which is non-toxic, for rats fed catechol along with molybdenum accumulate much more molybdenum than rats fed molybdenum alone, according to experiments of Nieland, Strong, and Elvehjem, reported by Comar.

Cunningham has tested the effects of tungsten, rhenium, zinc, manganese, vanadium, chromium, uranium, and tantalum on the intake of copper. So far, no effect of any of these has been discovered.

Certain organic compounds also affect the uptake or metabolism of copper. Cartwright has determined the values of plasma copper in various types of experimental anemia induced in swine. The plasma copper is significantly diminished in deficiencies of pyridoxine or of pteroylglutamic acid and protein, and it is reduced by practically one-half in simple protein deficiency. Iron deficiency does not affect the blood copper. The result of acute hemorrhage is to increase the blood copper considerably. In the pyridoxine deficiency and after hemorrhage the plasma iron and copper vary reciprocally, but that relation does not hold in protein deficiency or " PGA plus protein " deficiency. The significance of these relations is still in doubt. It seems too bad, in the light of our present knowledge of species differences in response to copper, molybdenum, and other elements, that more susceptible species, such as sheep or cattle, were not used instead of swine.

In summarizing this section and the preceding, one is impressed not only by the importance of molybdenum in the utilization of copper by mammals, but also by the profound differences between species, and even between breeds of the same species, in their responses to these micronutrients. Generalization seems particularly perilous because of the fact that beneath these factors of variability lie the equally significant variables of the mineral content of the forage, which in turn rest upon the innumerable varieties of soil. We must next consider these fundamentals.

Copper Metabolism in Plants

Current methods for producing micronutrient deficiencies in plants are reviewed by Steinberg, who emphasizes particularly the calcium carbonate method and the use of organic compounds such as oxine to form heavy metal complexes which can then be removed. Arnon has restated the criteria for regarding an element as essential to a plant:

"(1) a deficiency of it makes it impossible for the plant to complete the vegetative or reproductive stage of its life cycle; (2) such deficiency is specific only to the element in question, and can be prevented or correcting only by supplying this element; and (3) the element is directly involved in the nutrition of the plant, quite apart from its possible effects in correcting some unfavorable microbiological or chemical condition of the nutrient medium."

For the tomato, copper satisfies these criteria. Symptoms of a severe deficiency, ending in death, result when the amount of copper available to the plant is reduced below .0001 mg. This may be corrected by supplying .002 mg. of copper, but not by any of some 20 other elements tried. In nutrient solutions .02 p.p.m. of copper is sufficient for healthy growth; 2.0 p.p.m. is toxic. Spraying the leaves of young copper-deficient plants with copper sulfate (.02 p.p.m. of Cu) restores the normal growth of the roots as well as the shoots. This is specially noteworthy, for not only does it exclude possible effects of the root environment on the absorption of copper but shows also that the leaves and aerial parts of the plant are capable of absorbing physiologically significant amounts of copper.

The symptoms of copper deficiency are very constant and characteristic for a given species. Many of these have been described by Steinberg. Tomato plants are very stunted in growth, their leaves curl, no flowers are formed, and there is a distinctive bluish-green sheen to the foliage. Onions, on the other hand, develop thicker scales and a better color when copper is adequate but do not suffer so severely when it is low. A characteristic copper deficiency disease is "die-back" of citrus trees; and the "reclamation disease" of cereals is another.

Protein content seems always to be increased in these copper deficiency diseases. (This was questioned by Granick in the discussion following Steinberg's paper.) In fact, Steinberg feels that possibly copper alone of the trace elements is not concerned with the synthesis of protein. Inasmuch as symptoms of various mineral deficiencies may be produced by the liberation of amino acids in the tissues, he suggests that if copper is involved in protein synthesis, then a copper deficiency might lead to an accumulation of amino acids, as in the case of such a metal as manganese, which is definitely concerned with protein synthesis.

Copper is one of several metals that greatly improve the utilization of an ammonium source of nitrogen by plants. Arnon has suggested that this may be due to a compensation to the plant for the loss of the oxidative function of nitrate. Such indirect and nonspecific roles cannot be the reason why copper is an essential metabolite for plants. Its status as a micronutrient points rather to some catalytic function. The possible roles of the copper enzymes as terminal oxidases in respiration have already been discussed. It remains to consider the relation of copper to photosynthesis.

In leaves, about three-fourths of the copper is concentrated in the chloroplasts, mostly in organic combination. It has been found that in *Chlorella* antagonists of such copper enzymes as polyphenol oxidase inhibit photosynthesis even more than respiration. Since it has been shown that in the photolysis of water by isolated chloroplasts quinones can serve as hydrogen acceptors, it seems a promising theory to consider that if the quinones are the natural hydrogen acceptors in photosynthesis, the copper oxidases may be responsible for their oxidation from the corresponding phenolic compounds. At this symposium Arnon has reported for the first time that he has performed experiments which support the hypothesis that metals take part in the light reaction of photosynthesis. This reaction is strongly inhibited by o-phenanthroline and may be reactivated by copper or zinc, among the micronutrient metals. Ferrous iron is rather ineffective, a puzzling fact now explained by the discovery that under the conditions of the experiment it is oxidized to ferric iron. When this is prevented, ferrous iron is a good antagonist of phenanthroline. It may therefore be postulated that the metals act as they do by forming complexes with the inhibitor, just as ferrous iron is known to do with phenanthroline.

The phenanthroline inhibition of photosynthesis is also reversible by dialysis. This may be explained by the hypothesis that the inhibitor " blocks the reactivity of an organic metal complex which is essential for the light reaction of the chloroplasts." That potassium ethyl xanthate is also a strong inhibitor of the light reaction points to *copper* as the metal, for among the metals tried only copper is effective in reversing this inhibition. In Arnon's conservative phrase, it may be said that " the possibility cannot be excluded at this time that copper may prove to be at least one of the metals concerned in the light reaction of photosynthesis in green plants."

Copper and Soils

It is obvious that copper deficiency of animals depends upon the copper content of the plants they eat and that the copper deficiencies of plants depend upon the soils in which they grow. In his survey of the deficiencies of green plants, Steinberg mentioned their occurrence on peat soils in New York state. Similar organic soils in California, Europe, and South Africa are accompanied by like phenomena. On the reclaimed peat soils of Europe the so-called " reclamation disease " of cereals is found. The peat and muck soils of the Florida Everglades furnished the forage for the unhealthy cattle described by Davis. In all such areas, copper sulfate (added at about 100 lbs. per acre) is a spectacular remedy.

In New Zealand there are about 250,000 acres of drained and reclaimed peat land. This is where the simple copper deficiency and the molybdenum-complication called peat scours afflict cattle and sheep. Whereas, according to Cunningham, the dry grass of normal pastures contains about 11 p.p.m. of copper and the soil itself about 18 to 22 p.p.m., grasses on the peat soils range from 3 to 7 p.p.m., and the total copper in the soil is around 5 p.p.m. " Available " copper may be still less. Assays by the *Aspergillus niger* method proved to be reliable for copper-deficient sands, but grossly overestimate the copper available to green plants in peat soils. The soils on which animals suffered from simple copper deficiency were always very low in copper, but the organic soils where the animals were afflicted with the complex molybdenum-copper deficiency were not so low in copper. The copper-deficiency diseases in New Zealand could easily be remedied by supplying no more than 5 pounds of copper sulfate per acre per year as a top dressing in the autumn. This yields a fairly stable copper content in the pasture of approximately 10 p.p.m.

A very different approach to the analysis of these peat or muck organic soils and their copper relations is to be found in the work of Dawson and Nair. These workers have developed a method of analyzing the nature of the copper in a soil by measuring with a copper amalgam electrode the cupric ion activity when a soil suspension is titrated with copper sulfate. Two regions of the titration curves turn out to be buffered with respect to the cupric ion activity, and these are interpreted as being due to the presence of two primary copper complexes. When the soils are titrated against ammonia, four characteristic buffered regions appear that are attributable to secondary complexes resulting from reaction of the primary complexes with ammonia. In their present contribution, Dawson and Nair have done much to identify these complexes. Some protein-lignin combination is responsible for both sets of copper complexes. Copper reacts with R–SH and R–COOH groups, perhaps in the same molecule, to form the complexes. The two primary complexes contain a secondary and a tertiary aromatic amine, respectively. An $R\text{–}NH_2$ group is necessary for the formation of two of the secondary copper-ammonia-peat complexes. The primary copper complexes were found to contain 3 atoms of copper, 2 molecules of cysteine, and 1 aromatic amine group. These analyses show how the copper present in a peat soil may be combined into organic complexes that render the copper which is actually available for plant or animal use far less than might appear from chemical estimates of the copper present. Although the method which has been used may involve an undetermined error, the general lines of the conclusion nevertheless appear to be sound.

The peat soils lack *available* copper. On the other hand, many sandy, inorganic soils are poor in copper in an absolute sense. The soils of the South Australian seaboard, where the sheep studied by Marston suffer from copper deficiency, are arid calcareous and siliceous soils, much weathered, and deficient in cobalt as well as copper. Trumble has provided a broad survey of the relation of these mineral soils to the nutritive qualities of herbage plants. It was in this area that the necessity for molybdenum in leguminous nitrogen fixation was first discovered. All the effects of the trace elements here depend greatly on phosphate, of which there is an acute shortage in the South Australian soils. The successful growth of pasture grasses depends completely on sufficient nitrogen fixation by the legumes, and the legumes cannot grow well to start with unless ample phosphate is provided. Trumble has considered the importance of the geochemical composition of the materials from which the soils have been derived, the effects of concentration, soil pH, and ionic exchange on the availability of copper, the results of leaching and water-logging, and the effects of the soil microflora and the association of various plants in the community. Climatic effects and seasonal patterns are particularly important in shifting the period of maximum rainfall in relation to the growing season determined by light and temperature. Many of these variables have been related to zinc, manganese, or molybdenum nutrition, but very little of this background has been applied so far to the analysis of copper relations. In the growth of legumes, a copper deficiency affects chiefly the production of seed. It also seems to hinder the development of the perennial species more than that of annuals. Because of the search for obvious symptoms, many cases of trace element deficiency have been overlooked—so Trumble thinks. Actual field experiments, using a factorial experimental design, are needed. Yields must be analyzed so as to allow for a multiplicity of limiting factors and their interactions.

Another illuminating study of the relations between the factors affecting soils and the soil content of micronutrient elements was contributed by Beeson and Matrone. This survey involved a region marked by a cobalt, rather than copper, deficiency, but the nature of the analysis can well stand as a model for further work in copper-deficient areas.

Preliminary surveys determined the principal range plants eaten by cattle in the cobalt-deficient region of North Carolina, as well as the cobalt concentration (ca. 0.10 p.p.m.) necessary for healthy growth of the animals (cattle and sheep), and the local topography. When it was seen that the cobalt-deficient regions were mostly in the lowlands along the rivers and streams, a detailed study was made of the series of Pleistocene terraces, seven in number, along the Coastal

Plain. These terraces are now very irregular, and in places some of them are completely interrupted. Transects of the terraces were then made in the northern, central, and southern portions of the state, and three locations were sampled on each terrace along each transect. Since drainage of the soil appeared to be important, and the principal forage plants did not grow equally on both well-drained land and poorly drained land, two representative plants were picked, the reed or cane (*Arundinaria tecta*) for the poorly drained, and the broomsedge (*Andropogon virginicus*) for the well-drained areas. For comparison, broomsedge was also sampled each time from the same area as the reeds. The plant samples of foliage were analyzed in duplicate for their content of cobalt, copper, manganese, iron and phosphorus.

As expected, cobalt turned out to be less abundant in the vegetation growing on poorly drained soil, there being no significant difference between the two species of plants. Nor was any significant change in cobalt content found in relation to the marine terraces. Copper content, on the other hand, was found to be strongly affected by the difference of species, reeds exceeding broomsedge in copper content when growing together on the same area. On the well-drained land the effect of elevation was slight, but on poorly drained soils there was a large increase in copper content from the seaboard up to a maximum on the fifth terrace (the Sunderland), followed by a decline with further increase in elevation. This was true for all three regions, north, central, and south.

The overall results show "(1) an increase in the content of mineral as the age and elevation of the Coastal Plain terraces increase [for all except cobalt]; (2) a maximum level of one or more mineral elements at some intermediate terrace [copper and phosphorus] and (3) an effect of drainage [cobalt and iron, reciprocally] or species [iron and copper], or both." Geological reasons could be given for the high concentration of manganese toward the south and on the fifth terrace, but the same reasons seem inadequate to explain the maximum of copper on the fifth terrace. In any case, it is clear that a careful study of this kind may have considerable predictive value, besides enabling the complex interactions of the factors that affect mineral content of the plant forage to be analyzed in a far more meaningful way than when a single type of plant is analyzed for a single trace element irrespective of type of soil, drainage, geological history, etc.

The analysis of soils with respect to multiple trace elements, as well as the other climatic, topographic, geologic, and ecologic relations discussed in these papers by Trumble and Beeson, will make possible a new era, one of the development of lands naturally unproductive. In an age when the world's food problem is becoming increasingly acute as population continues to soar almost everywhere, few enterprises can offer more promise than this type of " environmental control," as Trumble has called it. This Copper Symposium represents a beginning in the synthesis of knowledge from many fields that is needed to make such an undertaking feasible.

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